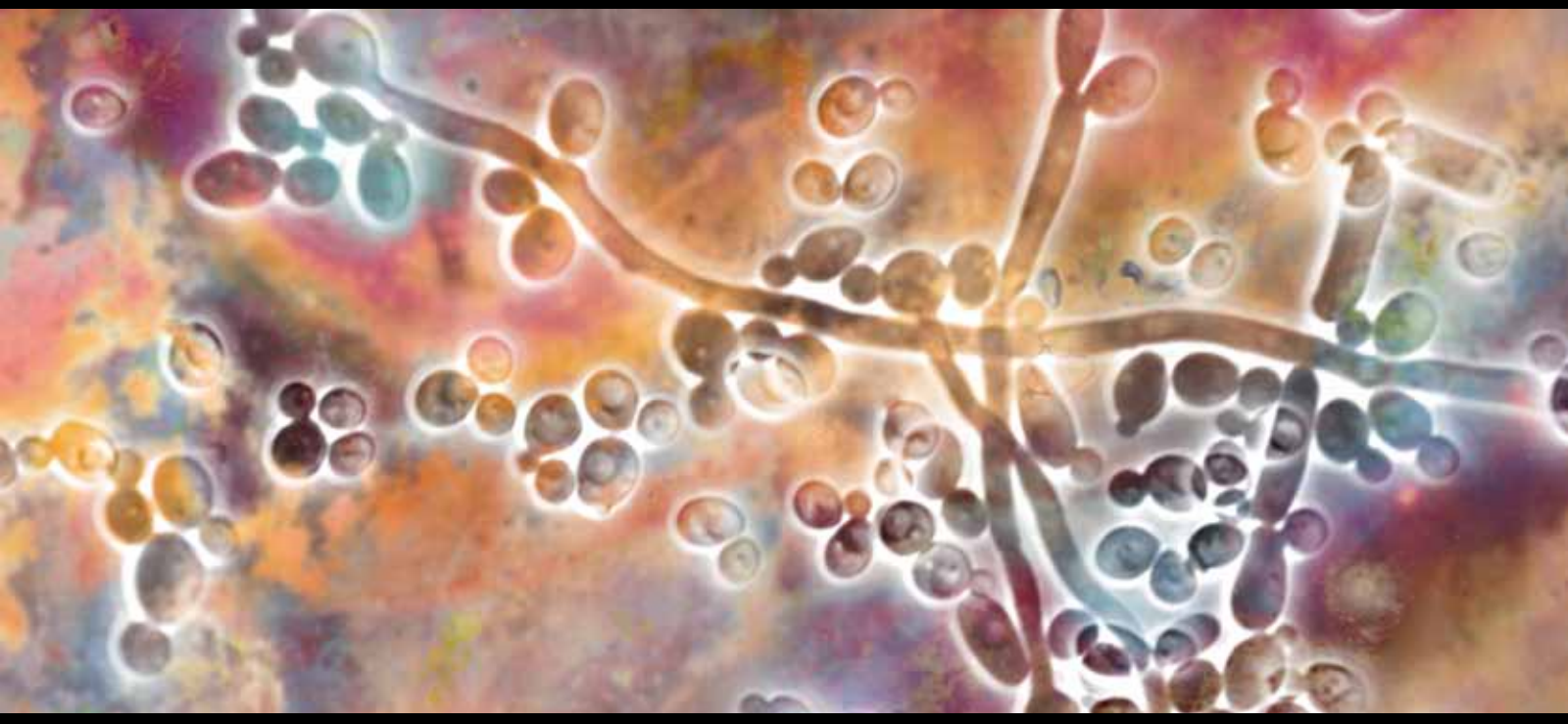
Interdisciplinary Perspectives on Infectious Diseases

# Role of Infection in Neurologic and Psychiatric Diseases

Guest Editors: Marylou V. Solbrig and Guey Chyen Perng



# Role of Infection in Neurologic and Psychiatric Diseases

Interdisciplinary Perspectives on Infectious Diseases

# Role of Infection in Neurologic and Psychiatric Diseases

Guest Editors: Marylou V. Solbrig and Guey Chuen Perng

# Contents

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 645834, 1 page
doi:10.1155/2010/645834

*Editorial*

# Role of Infection in Neurologic and Psychiatric Diseases

**Marylou V. Solbrig[1] and Guey Chuen Perng[2]**

[1] *Department of Medicine (Neurology) and Medical Microbiology, Health Science Center, University of Manitoba, Winnipeg, Manitoba, Canada R3A 1R9*
[2] *Department of Pathology and Laboratory Medicine, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA*

Correspondence should be addressed to Marylou V. Solbrig, msolbrig@uci.edu

Received 7 April 2010; Accepted 7 April 2010



What makes CNS injury by pathogens different from other CNS afflictions such as stroke or Alzheimer's Disease is well illustrated in the manuscripts of this volume. We will see that viral, bacterial, or prion-induced injuries are dynamic and varied. No two infection cases are the same due to host or agent factors. We will encounter pathogens that are shared with other species and some that range across the globe. We will also find robust examples of gene x age x environment interactions as determinants of nervous system disease, which can be incorporated into understanding many neurobiological processes.

The role of infection in neurologic and psychiatric diseases is predicated on clinical and research observations, which have been expanded into the domains of neurobiology by hypotheses from several perspectives. Our collected papers come from perspectives of epidemiology, protein biochemistry, immunology, genetics, molecular biology, pathology, behavioral sciences, psychology, neuropharmacology, and human and veterinary medicine. These articles are comprehensive reviews, topical summaries, or original research papers.

We hope that this collection of manuscripts provides a greater understanding of both familiar and unfamiliar pathogens, prompts new ideas on disease mechanisms and treatments, and stimulates further cooperative work on infection in neurologic and psychiatric diseases.

We thank our contributors from around the world. When it comes to transmissible diseases, the world is a small place. Although we did not specifically solicit for public health topics, we note that cross-species transfers, emerging diseases, epidemics and pandemics, are developed as key discussions in several of our manuscripts. For this reason we dedicate this special issue to the One Health Initiative, whose work is defined by the Mission Statement on their website: www.onehealthinitiative.com/mission.php "Recognizing that human and animal health and mental health.... are inextricably linked, One Health seeks to promote, improve, and defend the health and well-being of all species by enhancing cooperation and collaboration between physicians, veterinarians, and other scientific professionals...."

We express our sincere gratitude to all authors and reviewers for their time and effort, to Betsy Foxman, Editor-in-Chief for the invitation, and the staff at Hindawi Publishing for their assistance and support.

*Marylou V. Solbrig*
*Guey Chuen Perng*

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 262415, 18 pages
doi:10.1155/2010/262415

*Review Article*

# Towards an Understanding of the Herpes Simplex Virus Type 1 Latency-Reactivation Cycle

**Guey-Chuen Perng[1] and Clinton Jones[2]**

[1] *Department of Pathology and Laboratory Medicine, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA*
[2] *Department of Veterinary and Biomedical Sciences, The Nebraska Center for Virology, University of Nebraska-Lincoln, Fair Street at East Campus Loop, Lincoln, NE 68583-0905, USA*

Correspondence should be addressed to Clinton Jones, cjones@unlnotes.unl.edu

Received 8 August 2009; Accepted 30 November 2009

Academic Editor: Marylou V. Solbrig



Infection by herpes simplex virus type 1 (HSV-1) can cause clinical symptoms in the peripheral and central nervous system. Recurrent ocular shedding can lead to corneal scarring and vision loss making HSV-1 a leading cause of corneal blindness due to an infectious agent. The primary site of HSV-1 latency is sensory neurons within trigeminal ganglia. Periodically, reactivation from latency occurs resulting in virus transmission and recurrent disease. During latency, the latency-associated transcript (LAT) is abundantly expressed. LAT expression is important for the latency-reactivation cycle in animal models, in part, because it inhibits apoptosis, viral gene expression, and productive infection. A novel transcript within LAT coding sequences (AL3) and small nonprotein coding RNAs are also expressed in trigeminal ganglia of latently infected mice. In this review, an update of viral factors that are expressed during latency and their potential roles in regulating the latency-reactivation cycle is discussed.

## 1. Introduction

At least 90% of the population are infected with herpes simplex virus type 1 (HSV-1), and infection can cause a variety of disorders [1, 2]. Recurrent ocular HSV-1 is the leading cause of infectious corneal blindness in industrialized nations [3]. In a murine model, ocular infection appears to induce autoimmune disorders leading to corneal antigen destruction and stromal keratitis [4]. HSV-1 infections also cause gastrointestinal disorders, esophageal disorders, and approximately 25% of all genital herpes infections [5, 6].

HSV-1 is the most commonly identified cause of acute, sporadic viral encephalitis in the U.S. accounting for 10%–20% of all cases [7]. It is estimated that there are approximately 2,000 new cases per year in the U.S. HSV-1 and HSV-2 can cause acute necrotizing encephalitis in infants, children, and adults. Encephalitis due to HSV-2 in newborn infants is a widespread disease in the brain and commonly involves a variety of other organs in the body including skin, eyes, and lungs [8].

Herpes simplex virus- induced encephalitis (HSE) is characterized by severe destruction of temporal and frontal lobe structures, including limbic mesocortices, amygdala, and hippocampus. Without antiviral therapy, the mortality rate is as high as 70%, but even after antiviral therapy 20% of these patients die. Despite early treatment, chronic progressive tissue damage in magnetic resonance imaging can be found up to 6 months following the onset of symptoms. Approximately 2/3 of the HSE cases occur because of reactivation from latency [9], which explains why there is high morbidity and long-term complications despite antiviral treatment [10–12].

HSE is often associated with necrotic cell death resulting from virus replication and inflammatory changes secondary to virus-induced immune response [13]. However, there is not a perfect correlation between virus burden in the brain and the severity of histological changes and neurological symptoms. Furthermore, a small number of HSE patients are negative for HSV-1 DNA early in the course of infection suggesting that factors other than virus replication are

involved in pathogenesis. The finding that mice lacking toll like receptor 2 (TLR2) are less susceptible to HSV-1-induced encephalitis than wild type mice or mice lacking TLR4 implies that TLR-2-mediated cytokine responses are detrimental to the host [14]. In addition, two children with HSE lack the intracellular protein UNC-93B and have impaired interferon responses [15]. It appears that the ability of the host to appropriately respond to HSV-1 infections in the brain is crucial for preventing HSE.

## 2. The Latency-Reactivation Cycle

*2.1. The Latency-Reactivation Cycle Has 3 Distinct Steps.* Despite a vigorous immune response during acute infection, HSV-1 establishes latency in ganglionic sensory neurons, typically trigeminal ganglia (TG) or sacral dorsal root ganglia [16, 17]. Although TG is a primary site of latency following ocular, oral, or intranasal infection [18–20], latent HSV-1 can also be detected in human adult nodose ganglia and the vagus nerve [5, 6]. Up to 40% of sensory neurons can be latently infected [21–25]. HSV-1 genomic DNA has also been detected in the central nervous system of a significant percentage of humans [18, 26, 27].

The steps of the latency-reactivation cycle have been operationally divided into three major steps: establishment, maintenance, and reactivation (Figure 1). Establishment of latency includes entry of the viral genome into a sensory neuron and acute infection. Viral gene expression is then extinguished, with the exception of the latency-associated transcript (LAT). For further details regarding viral gene expression during acute infection and establishment of latency, see Section 2.2.

Maintenance of latency is a phase that lasts for the life of the host and is operationally defined as a period when infectious virus is not detected by standard virus isolation procedures. In general, abundant expression of viral genes that are required for productive infection does not occur. LAT is abundantly expressed during this stage of latency.

Reactivation from latency is initiated by external stimuli (stress and immunosuppression, e.g.) that stimulate viral gene expression. Abundant viral gene expression is detected in sensory neurons and infectious virus can be isolated from TG, eye swabs, and/or nasal swabs. It is not clear whether a neuron that undergoes reactivation and produces infections virus survives and resumes latency or is killed. For further discussion of factors that regulate reactivation from latency, see Sections 2.4–2.6. The ability of HSV-1 to reactivate from latency results in recurrent disease and virus transmission.

### *2.2. Viral Gene Expression during Productive Infection versus Latency*

*2.2.1. Viral Gene Expression during Productive Infection.* Binding and entry of HSV-1 to cells are mediated by viral glycoproteins and cellular factors [28–30]. A cellular mediator of viral entry (HveA or HVEM) is primarily expressed in activated T cells and belongs to the tumor necrosis factor receptor family [31]. Entry of HSV-1 into epithelial and other nonlymphoid cells is mediated by an unrelated membrane glycoprotein that resembles the poliovirus receptor (HveB and HveC) [32]. HveC is active as an entry mediator for all herpesviruses examined to date, HSV-1, bovine herpesvirus 1 {BHV-1}, and pseudorabies virus, {PRV}. HveC is abundantly expressed in neurons and can block viral entry in several neuron-like cell lines [32]. After uncoating, the viral genome is present in the nucleus and viral gene expression ensues.

HSV gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E), or late (L) [33]. IE transcription does not require protein synthesis and is stimulated by VP16 [34]. E gene expression is dependent on at least one IE protein, and generally E genes encode nonstructural proteins that play a role in viral DNA synthesis. L gene expression is maximal after viral DNA replication, requires IE protein production, and L proteins comprise the virion particle.

Five IE genes encode ICP0, ICP4, ICP22, ICP27, or ICP47. ICP4 [35–38] and ICP27 [39–41] are required for virus growth in tissue culture. In general, ICP4 represses IE gene expression [37, 42–46] and activates E or L gene expression by interacting with RNA polymerase II transcription factors [46, 47]. ICP27 redistributes small nuclear ribonucleoprotein complexes, interferes with splicing of IE transcripts, and promotes E and L poly A site selection [48–51]. ICP47 prevents transport of antigenic peptides into the endoplasmic reticulum [52] and is crucial for neurovirulence because it inhibits $CD8^+$ T cell responses [53]. ICP0 can activate expression of all classes of viral genes, in part because it increases steady-state levels of mRNA [54].

ICP0 also binds several cellular proteins: (1) elongation factor $1\alpha$ [55], (2) cyclin D3 [56], (3) an ubiquitin-specific protease [57, 58], and (4) PML [59–62]. Interactions between ICP0 and chromatin-remodeling enzymes activate viral transcription. For example, a histone deacetylase inhibitors enhance viral gene expression and productive infection [63, 64]. Secondly, ICP0 alters a complex that inhibits gene expression (REST/CoREST/histone deacytlase repressor complex) [65]. Finally, HSV-1 ICP0 interacts with HDAC2 [66] and blocks histone deacetylation to stimulate viral gene expression [65, 67]. Since ICP0 has recently been shown to remove histones from viral chromatin during productive infection [68], it is tempting to suggest that ICP0 has a similar function during reactivation from latency. These activities of ICP0 promote virus replication in differentiated cells [69].

*2.2.2. Viral Gene Expression Is Extinguished after Infection of Sensory Neurons.* Following infection of rodents, rabbits, or humans with HSV-1, productive infection is initiated in the mucosal epithelium. Virus particles or subparticles then enter sensory neurons and are transported intra-axonally to the sensory ganglia. Since HSV-1 infection typically occurs via the oral, ocular, or nasal route, the 5th cranial nerve, trigeminal ganglia (TG), is a primary site for latency [18, 19]. Extensive viral gene expression and replication occur within TG for approximately a week following infection of animal

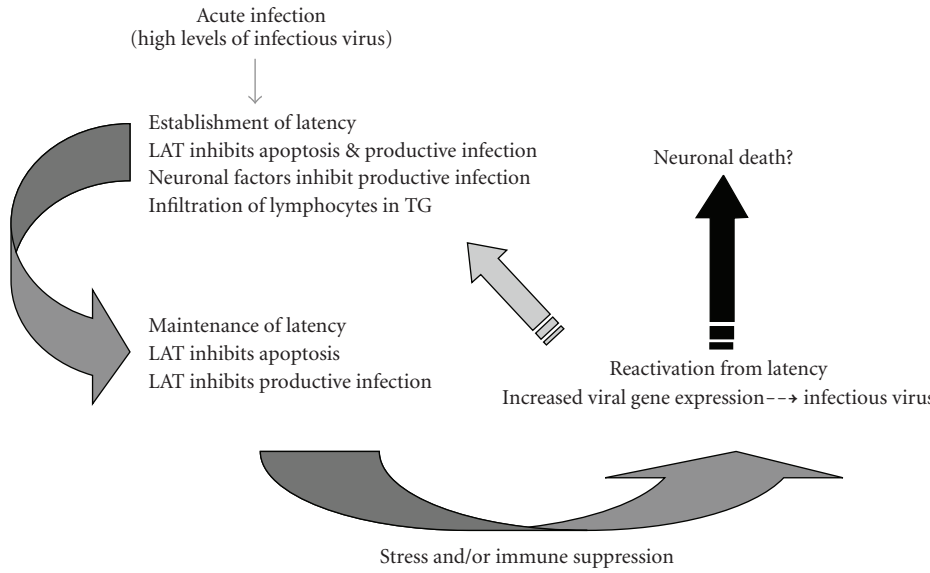



Figure 1: Steps in the latency-reactivation cycle of HSV-1. For details, see the text.

models that support HSV infection [70, 71]. Productive viral gene expression that occurs in TG appears to be different than what is seen in cultured cells [72]. Infectious virus can readily be detected in homogenates prepared from TG during acute infection. However, it is difficult to conclude whether this infectious virus is the result of productive infection in sensory neurons or the result of transport from peripheral sites of infection. Replication is not required for establishment of latency because mutants that cannot replicate will establish latency, but at a reduced level [73–81].

*2.2.3. IE Promoters Are Differentially Regulated in Sensory Neurons Relative to Nonneuronal Cell Types.* Several studies using transgenic mice that contain IE promoters linked to a reporter gene have concluded that IE promoters are differentially regulated by neuronal specific factors. For example, the HSV-1 ICP4 promoter is active in Schwann cells, but not sensory neurons in TG [82]. As expected, the ICP4 promoter in transgenic mice is activated in TG neurons following infection with HSV-1. In contrast to the ICP4 promoter, transgenic mice containing the ICP0 or ICP27 promoters are active in certain neurons within the brain and TG [83]. The ICP0 promoter is also differentially regulated in TG neurons depending on the age of the mouse. The ICP0 promoter contains a cis-acting element that can bind a neuronal specific transcription factor, Olf-1, which is differentially and developmentally expressed in specific subsets of sensory neurons [84] suggesting that the Olf-1 site plays a role in activating ICP0 promoter activity in certain neurons.

All IE promoters contain a common cis-acting sequence (TAATGARAT) that is required for VP16-mediated transactivation [34, 85]. VP16 must interact with two cellular proteins, Oct-1 and HCF, to efficiently induce IE promoter activity. A cellular transcription factor, Zhangfei, binds to HCF and prevents activation of the ICP0 promoter [86]. Another cellular transcription factor, Luman, also binds to HCF and sequesters HCF in the cytoplasm of sensory neurons, suggesting that Luman has a role in latency [86]. Zhangfei and Luman have basic domain-leucine zippers (bZIP) regions, acidic activation domains, and consensus HCF-binding motifs, yet have little amino acid similarity. In nonneuronal cells, HCF has a nuclear localization [87], but in sensory neurons it appears to be predominantly localized to the cytoplasm [88]. If the relative levels of Luman and Zhangfei are high, the availability of "free" HCF that could interact with VP16 would be reduced and consequently IE gene expression would be repressed. It has also been hypothesized that VP16 is not present in sufficient quantities in the nucleus of infected sensory neurons to stimulate efficient productive infection [88]. However, inducible expression of VP16 in the context of the viral genome or in transgenic mice did not lead to enhanced viral replication [89].

Other cellular transcription factors expressed in sensory neurons (Brn-3.0 and N-Oct3, e.g.) have the potential to regulate IE gene expression [90, 91]. Brn-3.0 binds to noncoding sequences in the HSV-1 genome, but the binding sites for Brn-3.0 are not identical to those for Oct-1 or other related transcription factors that also include Brn-3.1 and Brn-3.2 [92]. Brn-3.0 is important in the peripheral nervous system of mice because null mutations in the *brn*-3.0 locus result in neonatal death with defects in sensory ganglia and specific central nervous system nuclei [93, 94]. *brn*-3.2 is required for differentiation of certain retinal ganglion cells [95]. One study has concluded that Brn-3.1 and 3.2 have opposite effects on a target promoter [96]. Considering that the Brn3 family of transcription factors is expressed in the peripheral nervous system, these proteins may regulate HSV gene expression during the latency-reactivation cycle.

Following infection of primary neurons, ICP0 does not appear to accumulate in the nucleus of infected cells [97]. An independent study also concluded that the function of ICP0 is impaired in human neuronal-like cells because a nuclear structure (ND10) that ICP0 interacts with is different compared to nonneuronal cells [98]. The same neuronal-like cells do not support efficient viral replication, in part, because ICP0 expressing plasmids do not activate viral transcription efficiently. These studies argue that ICP0 does not function efficiently in neuronal cells and thus productive infection is inhibited.

*2.2.4. The LAT Promoter Is Neuronal Specific.* In sharp contrast to other HSV-1 promoters, the promoter that directs expression of the latency-associated transcript (LAT) is activated in sensory neurons (see Figure 2 for a schematic of the HSV-1 LAT promoter). Two separate promoter fragments that are upstream of the start site of LAT, latency-associated promoter 1 and 2 (LAP1 and LAP2), can cis-activate a reporter gene in transiently transfected cells [99, 100]. Several studies have demonstrated that sequences spanning the TATA box, LAP1, are critical for directing LAT expression in sensory neurons [99, 101–104]. LAP2 promoter has been proposed to promote expression of the stable 2 Kb LAT expression during productive infection of cultured cells. LAP2 may also play a role in promoting long-term expression of LAT in sensory neurons or may activate expression of novel transcripts during specific stages of infection in sensory neurons. Although the LAT promoter elements have neuronal specificity in transient transfection assays, they can also direct expression of a reporter gene in nonneural cells [105–109]. This may reflect the abundance of cellular transcription factor binding sites within the LAT promoter (Figure 2(c)). Many of these transcription factors are present in nonneural cells and can activate the LAT promoter in transiently transfected cells. For example, the two CRE binding sites in the LAT promoter are functional because cAMP activates the promoter [110, 111]. The CRE motif that is proximal to the TATA box is important for expression in neurons, and its presence has a positive effect on reactivation from latency [111–113]. Furthermore, Sp1, YY1, USF, and CAAT are frequently found in RNA polymerase II promoters that are not neural specific. Neuronal specific factors have been identified that bind to the LAT promoter [111–113]. The finding that the IE protein, ICP4, binds to DNA sequences downstream of the TATA box and represses the LAT promoter is one important reason why LAT is not an abundant transcript during productive infection [107].

Long-term expression of LAT has also been examined in the context of the viral genome [122–125]. These studies have demonstrated that LAP2 sequences function as a long-term enhancer (Figure 2(c)) in latently infected mice. LAP2 also appears to maintain LAP1 promoter activity. Although DNA sequences within the LAT promoter activate RNA expression in sensory neurons, neuronal specificity does not appear to be contained into a single cis-acting motif. As expected, the LAT locus is transcriptionally active during latency and is associated with acetylated histones, whereas ICP0 expression is repressed and hypoacetylated [126].

### *2.3. Viral Gene Expression Is Restricted during Latency to the LAT Locus*

*2.3.1. LAT Is Abundantly Expressed in Sensory Neurons during Latency.* LAT is abundantly transcribed in latently infected neurons of mice, rabbits, or humans [1, 104, 114, 115, 127–131]. Mice, rabbits, or humans latently infected with HSV-1 express LAT, and LAT is predominantly detected in the nucleus. LAT is complementary to ICP0 and overlaps the ICP0 transcript (Figure 2(b)), suggesting that LAT inhibits ICP0 expression by an antisense mechanism. Although the ability of LAT to repress ICP0 expression may be important, LAT sequences that promote spontaneous reactivation in a rabbit ocular model do not overlap ICP0 [132]. The simplest interpretations of these data are that LAT has more than one function or the ability of LAT to repress ICP0 expression is not that important in the small animal models used to study latency.

Detection of thymidine kinase and ICP4 transcripts, in addition to LAT, in TG of latently infected mice [133] appears to be the result of spontaneous reactivation or unsuccessful reactivation from latency [134, 135]. Viral genome positive neurons that are LAT negative can be detected in latently infected mice [24]. Since in situ PCR was used to detect viral DNA, but in situ hybridization was used to detect LAT, neurons expressing low levels of LAT were likely missed.

Splicing of the 8.5 Kb LAT transcript yields an abundant 2 Kb LAT and an unstable 6.5 Kb LAT [109, 114, 128] (Figure 3). Correct splicing of the 2 Kb LAT is necessary for establishment and maintenance of latency [139, 140]. In general, the stable 2 Kb LAT is not capped, is poly A-appears to be circular, and is a stable intron [141, 142]. Although LAT is predominantly detected in the nucleus, it is also present in the cytoplasm [143–145] and is associated with polyribosomes [143, 146] or splicing factors [143].

*2.3.2. Small Nonprotein Coding RNAs Are Encoded within the LAT Locus.* Small noncoding RNAs can regulate gene expression [147, 148], promote neuronal differentiation [149], or inhibit apoptosis [150]. There are numerous types of small noncoding RNA: short interfering (si) RNA [151], small temporal RNA [152], heterochromatic siRNA [153], tiny noncoding RNAs [154], and micro-RNAs (miRNAs) [155]. miRNAs are nonprotein coding RNA molecules that are synthesized in the nucleus as 70–90 nucleotide precursors, and then processed into 21–23 nucleotide single-stranded RNA by the Dicer nuclease in the cytoplasm [155]. Dicer also processes siRNA. Following the discovery of 5 miRNAs encoded within the Epstein-Barr virus genome [156], miRNAs have been identified in Kaposis sarcoma-associated virus [157–159], mouse gammaherpesvirus 68 [157], human cytomegalovirus [157, 160, 161], HSV-1 [156, 162], Marek's disease virus [163], and simian virus (SV40) [164].

A study by Umbach et al. [136] concluded LAT is a miRNA precursor that encodes four miRNAs, and two within LAT promoter sequences (Figure 3(a)). One of these miRNAs, LAT miR-H6, inhibits ICP4 protein levels but not ICP4 RNA levels. ICP0 protein levels, but not RNA levels,

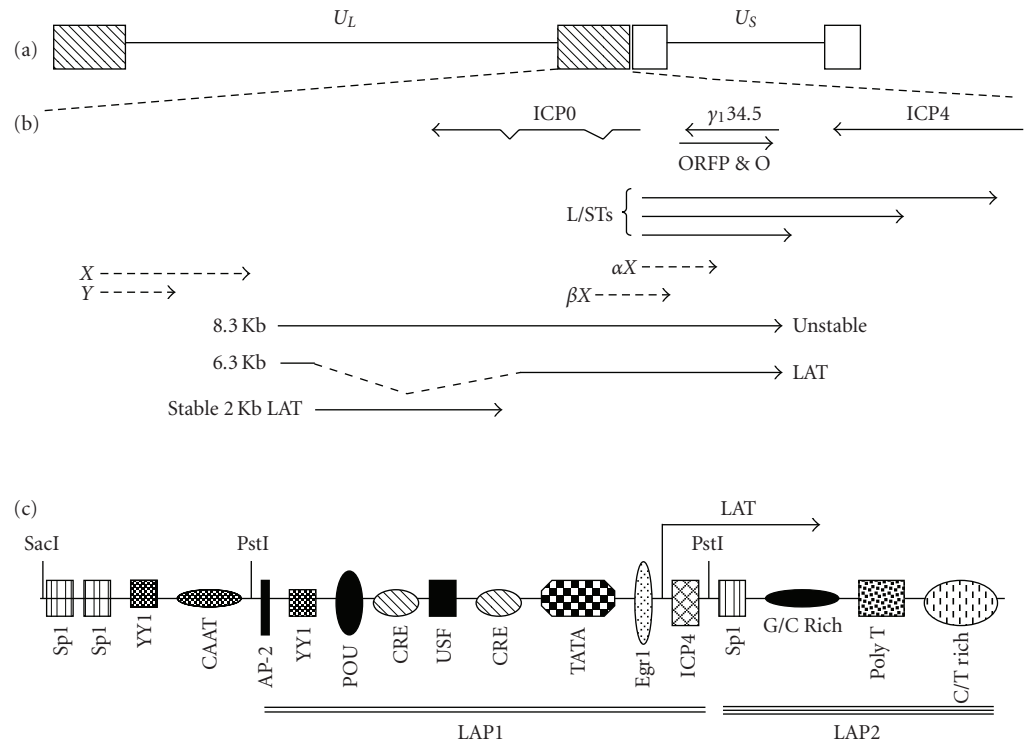



Figure 2: Location of genes within the HSV-1 repeats. (a) $U_L$ and $U_S$ denote the unique sequences of the long ($L$) and short ($S$) components of the genome. The boxes depict repeat sequences. (b) Transcription map of the repeat region. Location and orientation of LAT [114, 115], ICP0, $\gamma$134.5 [116, 117], ORFP [118], L/STs [119] are indicated by solid lines. Partially mapped transcripts ($\alpha$X and $\beta$X) are denoted by dashed arrows [120, 121]. (c) The LAT promoter contains numerous cis-acting sites that can be bound by cellular transcription factors. Binding of ICP4 to the ICP4 binding site in the LAT promoter inhibits promoter activity [107]. In transient transfection assays, the LAT promoter can be divided into a strong promoter (LAP1) and a weaker promoter (LAP2) [99, 100]. For details of transcripts encoded by LAT, see Figure 3.

are inhibited by another LAT miRNA, miR-H2-3p. The authors conclude that suppression of ICP0 and ICP4 by these miRNAs "facilitates the establishment and maintenance of viral latency." Since the six LAT-specific miRNAs are not located within the first 1.5 kb of LAT coding sequences, they may only play a supportive role during the latency-reactivation cycle in small animal models of infection. The fact that LAT-specific miRNAs inhibit ICP0 or ICP4 suggests that they enhance the establishment or maintenance of latency. In the context of the latency-reactivation cycle in small animal models, it is unlikely, they are crucial when compared to the first 1.5 kb of LAT coding sequences.

Two additional small RNAs (s-RNAs) are encoded within the first 1.5 kb of LAT coding sequences (LAT s-RNA1 and s-RNA2) [138] (Figure 3(b)). Expression of LAT s-RNA1 and s-RNA2 is readily detected in trigeminal ganglia of latently infected mice [165]. LAT s-RNA2 inhibits ICP4 protein expression, but not RNA expression. LAT s-RNA1 inhibits productive infection approximately 1,000-fold in transient transfections assays, whereas LAT s-RNA2 only inhibits productive infection 5-fold [165]. These LAT s-RNAs may not be miRNAs because they lack Dicer cleavage sites and a mature miRNA band that migrates between 21 and 23 nucleotides was not detected. LAT s-RNA1 and s-RNA2 would not have been identified using the methods described by Umbach et al. [136] because they size selected RNA species migrating between 17 and 30 nucleotides, and LAT s-RNA1 is 62 nt long and LAT s-RNA2 is 36 nt long.

*2.3.3. Novel Transcripts Are Expressed within LAT Coding Sequences.* Sequences that encompass LAT also encode several additional transcripts. For example, novel transcripts within the LAT promoter region have been reported [166]. More recently, a transcript and protein, UOL (Upstream of LAT), was identified that is encoded within the LAT promoter regulatory region [167]. Deletion of UOL does not dramatically reduce the spontaneous reactivation phenotype in rabbits [168]. Another transcript, antisense to LAT (AL), is expressed within the first 1.5 kb of LAT coding sequences and the start site of the LAT promoter and appears to encode a protein [169] (see Figure 3 for location of UOL and AL).

Two small ORFs that are also antisense to LAT (AL2 and AL3) have been identified within the first 1.5 kb of LAT coding sequences (Figure 3(b)). A transcript within the first 1.5 kb of LAT coding sequences (AL3) is expressed during productive infection and in trigeminal ganglia of latently infected mice [170]. Like AL, AL3 is antisense with respect to LAT. An AL3 protein was also detected in cells transfected with an AL3 expression vector, and in trigeminal ganglia of infected mice. Conversely, an AL3 protein was not detected

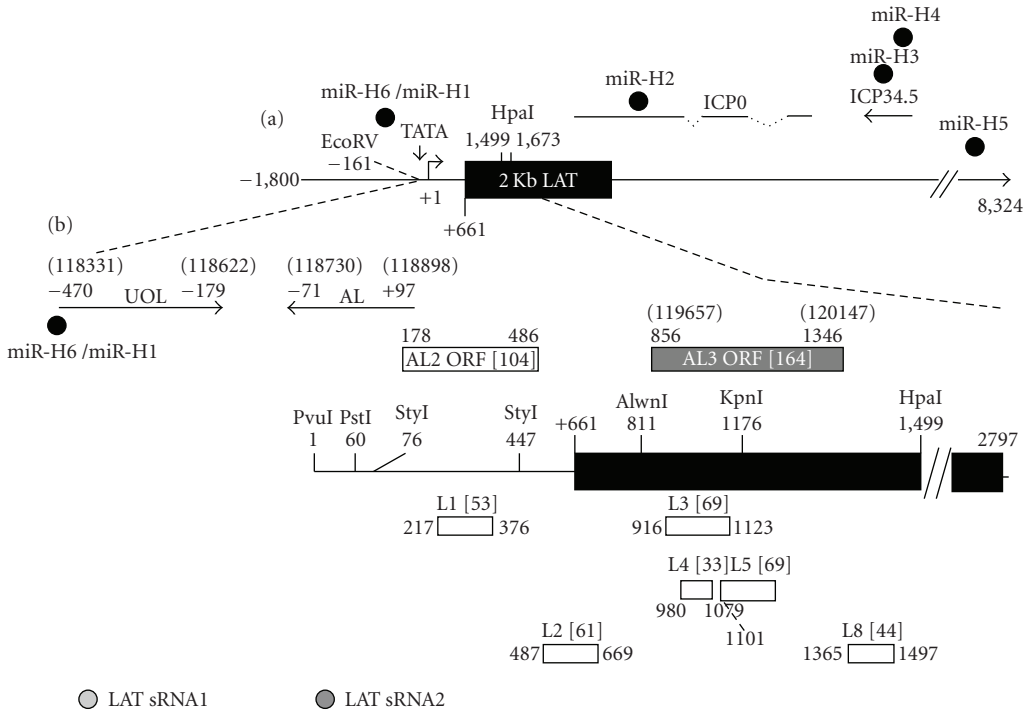



Figure 3: Schematic of factors encoded within the LAT locus. (a) Schematic of genes within the long repeats that contain the LAT locus. The large arrow indicates the primary LAT transcript. The solid rectangle represents the very stable 2 kb LAT intron. The start of LAT transcription is indicated by the arrow at +1 (genomic nucleotide 118801). Several restriction enzyme sites and the relative locations of the ICP0 and ICP34.5 transcripts are shown for reference. The locations of the 6 micro-RNAs (miR-H1-6) that are located within the 8.3 kb LAT [136] are shown. (b) Partial restriction map of LAT and position of LAT open reading frames (L1-8) within the first 1.5 Kb of strain McKrae LAT coding sequences, which were based on previous studies [137]. The numbering system of the ORFs was consistent with a previous study [137]. Only the ORFs with at least 30 amino acids are shown (the number of amino acids in each ORF is denoted by the numbers in brackets). Open circles denote the position of two LAT small RNAs that are encoded within the first 1.5 kb LAT coding sequences [138]. Positions of UOL transcript, AL transcript, and ORFs located on the opposite strand of LAT (AL2 and AL3) are shown. The numbers of amino acids of AL2 and AL3 are in brackets. Nucleotide positions relative to the start of LAT transcription are not shown in parenthesis. Numbers in parentheses represent HSV-1 nucleotide positions.

during productive infection, in part, because the 5′ terminus of the AL3 transcript is downstream of the first in frame methionine of AL3. It is not currently known whether a transcript encompassing AL2 is expressed during productive infection or during latency. It remains to be seen whether AL2 or AL3 plays a role in the latency-reactivation cycle of HSV-1.

*2.4. LAT Regulates the Latency-Reactivation Cycle.* As discussed above, the latency-reactivation cycle of HSV-1 can be operationally defined in 3 steps: establishment of latency, maintenance of latency, and reactivation from latency (summarized in Figure 1). In a human being, latency is maintained for the life of the host, indicating that a well-conceived strategy exists that allows for periodic reactivation, while maintaining the viral genome in sensory neurons.

Numerous HSV-1 mutants that do not express detectable levels of LAT have been constructed and tested in animal models [17, 171]. Although a couple of studies have suggested that LAT plays no role in a latent infection [172, 173], most have concluded that LAT is important but not required. LAT enhances establishment of latency in mice [174, 175] because certain LAT-mutants contain lower levels of viral DNA in murine TG relative to wild type virus [21, 176]. Furthermore, LAT enhances establishment of latency in the rabbit eye model and consequently reduces reactivation from latency [177]. The finding that LAT represses productive viral gene expression in TG of mice during acute infection [178, 179] supports the studies concluding that LAT facilitates establishment of latency. When considering the role that LAT plays in reactivation from latency, its role in establishing latency must be taken into consideration.

LAT enhances establishment of latency in mice [174, 175] or rabbits [180] because certain LAT mutants contain lower levels of viral DNA in TG relative to wt virus [21, 176]. LAT represses productive viral gene expression in TG of mice during acute infection [178, 179] supporting the concept that LAT facilitates establishment of latency. The HSV-1 McKrae strain is frequently shed in tears of infected rabbits as a result of spontaneous reactivation, and LAT is crucial for spontaneous reactivation [177, 181–184]. Furthermore, HSV-1 17syn+ strains with deletions in LAT coding sequences do not reactivate efficiently using the

rabbit eye model [185, 186]. Although LAT overlaps the ICP0 transcript, LAT sequences that promote the latency-reactivation cycle in rabbits do not overlap ICP0 [132].

LAT is also important for in vivo reactivation using two different rabbit eye infection models. The McKrae strain of HSV-1 is frequently shed in the tears of infected rabbits as a result of spontaneous reactivation [177, 181, 183, 184, 187]. In contrast, spontaneous reactivation is severely impaired if the LAT gene is deleted. However, these same LAT-mutants grow with the same efficiency as wild-type virus in cultured cells and in ocular tissue of infected rabbits. The first 1.5 Kb of the gene encoding LAT is sufficient for spontaneous reactivation from latency [177] (Figure 3). Since this region does not overlap ICP0, antisense repression of ICP0 expression by LAT does not appear to be required for spontaneous reactivation in the rabbit model. HSV-1 17syn+ strains that have deletions in the LAT promoter and 5′ region of the gene encoding LAT (approximately 1,200 base pair) also do not reactivate efficiently in a rabbit eye model [185, 186].

It is not clear whether LAT encodes a protein that regulates the latency-reactivation cycle. Although certain studies suggested that LAT does not encode a protein [137], several studies have concluded that a protein encoded within LAT sequences is expressed [118, 167, 188–192]. These proteins were suggested to substitute for ICP0 functions [191, 192], interfere with binding of ICP4 to DNA [190], or their functions were not described. These proposed LAT proteins map downstream of the critical first 1.5 kb of the primary LAT transcript, a region that appears both sufficient and necessary for LAT's antiapoptosis activity and its ability to support a wild type spontaneous reactivation phenotype [177, 193]. Within the first 1.5 kb of LAT coding sequences, 8 potential ORFs have been identified in the strain McKrae [137] (summarized in Figure 3(b)). A recent study has provided evidence that L2 (Figure 3(b)), which is located in the first 1.5 kb of LAT coding sequences, appears to be expressed in TG of latently infected mice [194]. In summary, the gene encoding LAT does not appear to be absolutely required for latency in small animal models. However, the importance of LAT may be underestimated using small animal models and measuring latency in terms of weeks or months, not decades. The involvement of a LAT encoded protein in the latency-reactivation cycle is unclear.

### 2.5. HSV-1 Encodes Several Genes That Regulate Apoptosis

*2.5.1. Genes Expressed during Productive Infection Inhibit Apoptosis.* Many viruses induce apoptosis in cultured cells [195–198]. Killing of infected cells by apoptosis in vivo can reduce inflammation, alter immune recognition, reduce burst size, and thus prevent virus spread. Members of the *Alphaherpesvirinae* subfamily induce apoptosis after infection of cultured cells [199–202]. HSV-1 can also induce or inhibit apoptosis in a cell type dependent manner after infection of cultured cells [200, 201, 203–205]. Several antiapoptotic genes encoded by HSV-1 (ICP27, $U_s3$, $U_s5$, gJ, gD, and LAT) have been identified [200, 201, 203, 204, 206–212]. $U_s3$ is a protein kinase that, in the absence of other HSV-1 proteins, inhibits cleavage of BAD and formation of the proapoptotic form of BAD. $U_s3$ is the only viral protein required for preventing caspase 3 activation, which is the "point of no return" following apoptosis induction. The presence of several HSV-1 antiapoptotic genes suggests that they have specific roles following infection of humans.

HSV infection can induce apoptosis by several distinct mechanisms. For example, HSV induces DNA damage, even in the absence of productive infection [213–217]. DNA damage is a potent stimulus for apoptosis [217]. When expressed from baculovirus expression vectors, $U_s1.5$ and $U_L13$ can activate caspase 3 [218]. As expected, $U_s3$ can inhibit the proapoptotic activity of $U_s1.5$ and $U_L13$ because it can interfere with caspase 3 activation.

*2.5.2. LAT Inhibits Apoptosis.* LAT interferes with apoptosis in transiently transfected cells and TG of infected mice or rabbits [139, 219–221]. LAT expressing plasmids inhibit caspase 8- and caspase 9-induced apoptosis [222, 223], the two major apoptotic pathways in mammals [224–226]. LAT also inhibits caspase 3 activation [227]. The antiapoptosis functions of LAT correlate with promoting spontaneous reactivation [219, 222]. In fact, inhibiting apoptosis appears to be the most important function of LAT because three different antiapoptosis genes [228–231] restore wt levels of spontaneous reactivation to a LAT null mutant.

LAT s-RNA1 and s-RNA2 (Figure 3) cooperate to inhibit cold-shock-induced apoptosis in transiently transfected mouse neuroblastoma cells [165]. Introduction of ATG → TTG mutations in ORFs within the first 1.5 kb of LAT coding sequences impairs the antiapoptotic functions of LAT [232] suggesting that LAT encodes a functional protein or alters RNA structure. Two of these ATG → TTG mutations are within LAT sRNA1 and sRNA2, and introducing these mutations into both small RNAs inhibits their ability to inhibit apoptosis [165]. Although this suggests that the LAT sRNAs mediate the antiapoptotic functions of the first 1.5 kb of LAT coding sequences, there may be additional functions within this region that have antiapoptosis functions.

### 2.6. Model Describing How LAT Regulates the Latency-Reactivation Cycle.

Based on published studies, a working model has been devised to explain how LAT regulates the latency-reactivation cycle. During acute infection of TG (1–4 dpi), extensive viral gene expression occurs [70–72]. The toxic effects of HSV-1 infection, in particular ICP0 [233, 234], $U_S1.5$, and $U_L13$ [218], make neurons vulnerable to damage and death. The ability of HSV to induce DNA damage [213, 215, 216, 235] would also stimulate the mitochondrial pathway of apoptosis [217]. The antiapoptotic properties of $U_S3$, $U_S5$, gD, gJ, ICP27, and LAT would promote neuronal survival during acute infection [139, 165, 200, 201, 203, 204, 209, 219–223]. Deletion of LAT might not have a dramatic effect on apoptosis frequency during the early stages of acute infection because the other viral antiapoptotic genes are expressed.

During transition from acute infection to latency (establishment of latency), viral gene expression is extinguished.

The ability of the LAT micro-RNAs to inhibit ICP0 and ICP4 proteins expression [136] as well as the ability of LAT sRNA1 and LAT sRNA2 to inhibit productive infection [165] are likely to promote the establishment of latency. Furthermore, LAT would be the only viral antiapoptotic gene abundantly expressed during the establishment of latency. Neurons in which extensive viral gene expression had occurred during acute infection (permissive neurons) would be vulnerable to apoptosis in the absence of LAT expression. Nonpermissive neurons that harbor viral genomes would have suffered low levels of viral induced damage and thus would have a higher probability of survival in the absence of LAT. In mice, subsets of neurons have been identified in TG and the ability of HSV-1 to infect these neurons is different [236], supporting the concept that permissive and nonpermissive neurons exist.

The antiapoptosis functions of LAT would also appear to be crucial for protecting neurons from apoptotic stimuli during the maintenance of latency because it is the only viral gene that is abundantly expressed (Figure 1). In fact, during latency, LAT does have an effect on the number of surviving neurons following infection of mice [237]. Furthermore, the ability of LAT micro-RNAs [136] and LAT sRNA1 or LAT sRNA2 [165] to inhibit viral gene expression and/or productive infection would promote maintenance of latency. Since LAT sRNA1 and LAT sRNA2 [165] are located within the first 1.5 kb of LAT coding sequences, these small RNAs appear to be more important than the LAT micro-RNAs. However, the ability of the respective LAT noncoding RNAs to inhibit viral gene expression or productive infection is not as important as inhibiting apoptosis because three different antiapoptosis genes restore wt levels of spontaneous reactivation to a LAT null mutant [228–231].

The response of the central or peripheral nervous system to trauma, stress, or immunosuppression plays an important role during reactivation from latency. Stress leads to elevated corticosteriod levels, which has rapid effects on neural activity [238, 239]. Dexamethasone, a synthetic corticosteriod, induces viral gene expression [240], stimulates an HSV-1 origin of replication (Ori-L) in neuronal cells [50], and alters splicing patterns in the absence of protein synthesis [241]. Corticosteroids, or other forms of stress or trauma can induce neuronal neurodegeneration and/or apoptosis [242–248]. Since reactivation induces productive gene expression, all HSV-1 antiapoptotic genes would be expressed and should prolong neuronal survival, thus enhancing virus production.

### 2.7. Cell-Mediated Immune Responses Are Important for the Latency-Reactivation Cycle

*2.7.1. Infiltration of Lymphocytes to TG during Acute Infection.* Several independent studies have demonstrated that T cells, $CD8^+$ T lymphocytes in particular, are crucial for controlling HSV infection in sensory ganglia [249, 250]. During acute infection, HSV antigen expression increases until 3 dpi in TG but is undetectable at 7 dpi [251]. Coincident with a decline of HSV antigen in TG there is an increase in Mac-1+ cells, macrophages, natural killer cells (NK), and certain $CD8^+$ cells. No cells with characteristic lymphoid cell morphology can be detected in uninfected TG. After 5 dpi, the number of $CD8^+$ T cells, F4/80+ cells (macrophages), and $\gamma\delta$ T cells increases dramatically. At 3 dpi, TG neurons that are viral antigen positive can be detected that are surrounded by nonneural cells expressing TNF-$\alpha$, IL-6, or IFN-$\gamma$ [252]. Cells that express IL-2 or IL-4 are detected later after infection when viral antigens are difficult to detect. The number of cells producing IFN-$\gamma$ and IL-4 increases between 3 and 7 dpi but the same cells do not appear to produce both factors [251]. At 7 days after infection, transcripts encoding IL-2, IL-10, IFN-$\gamma$, TNF-$\alpha$, or RANTES (regulated upon activation, normal T cell expressed and secreted mRNA) are detected by RT-PCR [253]. By ELISA, IL-2, IL-6, IL-10, and IFN-$\gamma$ are detected at the same time confirming the RT-PCR results. The same cellular antigens were not detected in TG from uninfected mice indicating that these changes were induced by infection.

*2.7.2. Persistence of Lymphocytes in the Peripheral Nervous System during Latency.* If true latency of HSV is established, cytokine expression in TG would not be detected. However, several studies have concluded that a persistent cell-mediated immune response occurs in TG during latency, and that T cells, $CD8^+$ T lymphocytes in particular, inhibit reactivation from latency [249–251, 254–258].

The obvious explanation for persistence of immune effecter cells in TG is that low levels of viral proteins are expressed and an immune response occurs. A careful examination of TG neurons for viral gene expression in HSV-1 latently infected mice (37–47 days after infection) demonstrated that abundant viral transcripts, viral protein, and viral DNA replication occur in approximately 1 neuron per 10 TG [259]. Infectious virus is not detected in these mice confirming that they were latently infected. Neurons expressing high levels of HSV-1 transcripts are invariably surrounded by foci of infiltrating white blood cells. The term "spontaneous molecular reactivation" has been coined to describe these rare neurons [259].

*2.7.3. Interferon Can Inhibit Reactivation from Latency.* Persistence of the immune system in TG during latency is believed to play a role in the latency-reactivation cycle. $CD8^+$ T cells that produce interferon-$\gamma$ play an important role in preventing reactivation from latency in sensory neurons in mice latently infected with HSV-1 [256, 257]. Two independent studies have also concluded that interferon-$\alpha$ and interferon-$\gamma$ control recurrent herpetic lesions [260, 261]. In addition to interferon, lymphocyte-mediated cytotoxicity could inhibit virus spread in TG. Lymphocyte-mediated cytotoxicity induces two potent apoptotic pathways: the granule exocytosis and the Fas-Fas ligand pathways [262, 263]. The granule exocytosis pathway is employed predominantly by $CD8^+$, natural killer, and lymphokine-activated killer cells. A recent study has demonstrated that release of granzyme B from $CD8^+$ T cells into latently infected neurons helps to inhibit reactivation from latency by cleaving ICP4 [264]. Since it is well established that granzyme B activates

caspase 3 and the intrinsic pathway of apoptosis [265], the ability of LAT to inhibit apoptosis during maintenance of latency appears to be important.

## 3. Conclusions

HSV-1 latency is a complicated virus host interaction that is crucial for virus transmission, survival in nature, and recurrent disease. Numerous studies have indicated that sensory neurons are the primary site for latency. Since LAT is abundantly expressed in latently infected neurons, it is not surprising to find that LAT is important for the latency-reactivation cycle in small animal models. It is currently not clear whether expression of a LAT protein is important. Given the fact that several LAT small RNAs, including 6 known micro-RNAs, are expressed during latency implies that these small nonprotein coding RNAs are important for life-long ltency in humans. The finding that LAT sRNA1 and sRNA2 cooperate to inhibit apoptosis and also can inhibit productive infection supports a regulatory role for these small RNAs during the latency-reactivation cycle. It is also possible that additional transcripts encoded within LAT coding sequences (AL, AL3, or UOL) play a role in the latency-reactivation cycle. It will be necessary to design viral mutants that do not express these respective factors and then test the ability of these viruses to reactivate from latency in small animal models of infection.

## Acknowledgments

The laboratory of C. Jones is supported by two USDA Grants (08-00891 and 09-01653), a PHS Grant (R21AI069176), and in part by a PHS Grant (1P20RR15635) to the Nebraska Center for Virology. Dr. Guey Chuen Perng's laboratory is supported by PHS Grant NINDS/NS049556.

## References

[1] K. D. Croen, J. M. Ostrove, L. J. Dragovic, J. E. Smialek, and S. E. Straus, "Latent herpes simplex virus in human trigeminal ganglia. Detection of an immediate early gene "anti-sense" transcript by in situ hybridization," *The New England Journal of Medicine*, vol. 317, no. 23, pp. 1427–1432, 1987.

[2] A. J. Nahmias and B. Roizman, "Infection with herpes-simplex viruses 1 and 2. 3," *The New England Journal of Medicine*, vol. 289, no. 15, pp. 781–789, 1973.

[3] A. B. Nesburn, *Report of the Corneal Disease Panel: Vision Research- a National Plan, 1983–1987 Part III*, Mosby Co., Saint-Louis, Mo, USA, 1983.

[4] Z.-S. Zhao, F. Granucci, L. Yeh, P. A. Schaffer, and H. Cantor, "Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection," *Science*, vol. 279, no. 5355, pp. 1344–1347, 1998.

[5] R. M. Gesser and S. C. Koo, "Latent herpes simplex virus type 1 gene expression in ganglia innervating the human gastrointestinal tract," *The Journal of Virology*, vol. 71, no. 5, pp. 4103–4106, 1997.

[6] J. M. Lohr, J. A. Nelson, and M. B. A. Oldstone, "Is herpes simplex virus associated with peptic ulcer disease?" *The Journal of Virology*, vol. 64, no. 5, pp. 2168–2174, 1990.

[7] L. Corey, "Herpes simplex virus," in *Principles and Practice of Infectious Diseases*, G. L. Mandell, J. E. Bennett, and R. Dolin, Eds., pp. 1762–1780, Elsevier/Churchill Livingston, Philadelphia, Pa, USA, month 2005.

[8] L. R. Stanberry, M. N. Oxman, and A. Simmons, "Herpes simplex viruses," in *Infectious Dieseases*, S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow, Eds., pp. 1905–1917, Lippincott Williams and Wilkins, Philadelphia, Pa, USA, 2004.

[9] S. Yamada, T. Kameyama, S. Nagaya, Y. Hashizume, and M. Yoshida, "Relapsing herpes simplex encephalitis: pathological confirmation of viral reactivation," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 74, no. 2, pp. 262–264, 2003.

[10] E. Lahat, J. Barr, G. Barkai, G. Paret, N. Brand, and A. Barzilai, "Long term neurological outcome of herpes encephalitis," *Archives of Disease in Childhood*, vol. 80, no. 1, pp. 69–71, 1999.

[11] N. McGrath, N. E. Anderson, M. C. Croxson, and K. F. Powell, "Herpes simplex encephalitis treated with acyclovir: diagnosis and long term outcome," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 63, no. 3, pp. 321–326, 1997.

[12] B. Skoldenberg, "Herpes simplex encephalitis," *Scandinavian Journal of Infectious Diseases, Supplement*, vol. 23, no. 80, pp. 40–46, 1991.

[13] R. L. DeBiasi, B. K. Kleinschmidt-DeMasters, S. Richardson-Burns, and K. L. Tyler, "Central nervous system apoptosis in human herpes simplex virus and cytomegalovirus encephalitis," *Journal of Infectious Diseases*, vol. 186, no. 11, pp. 1547–1557, 2002.

[14] E. A. Kurt-Jones, M. Chan, S. Zhou, et al., "Herpes simplex virus 1 interaction with toll-like receptor 2 contributes to lethal encephalitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 5, pp. 1315–1320, 2004.

[15] A. Casrouge, S.-Y. Zhang, C. Eidenschenk, et al., "Herpes simplex virus encephalitis in human UNC-93B deficiency," *Science*, vol. 314, no. 5797, pp. 308–312, 2006.

[16] C. Jones, "Alphaherpesvirus latency: its role in disease and survival of the virus in nature," *Advances in Virus Research*, vol. 51, pp. 81–133, 1998.

[17] E. K. Wagner and D. C. Bloom, "Experimental investigation of herpes simplex virus latency," *Clinical Microbiology Reviews*, vol. 10, no. 3, pp. 419–443, 1997.

[18] J. R. Baringer and P. Pisani, "Herpes simplex virus genomes in human nervous system tissue analyzed by polymerase chain reaction," *Annals of Neurology*, vol. 36, no. 6, pp. 823–829, 1994.

[19] J. R. Baringer and P. Swoveland, "Recovery of herpes-simplex virus from human trigeminal ganglions," *The New England Journal of Medicine*, vol. 288, no. 13, pp. 648–650, 1973.

[20] F. O. Bastian, A. S. Rabson, C. L. Yee, and T. S. Tralka, "Herpesvirus hominis: isolation from human trigeminal ganglion," *Science*, vol. 178, no. 4058, pp. 306–307, 1972.

[21] J. Maggioncalda, A. Mehta, Y. H. Su, N. W. Fraser, and T. M. Block, "Correlation between herpes simplex virus type 1 rate of reactivation from latent infection and the number of infected neurons in trigeminal ganglia," *Virology*, vol. 225, no. 1, pp. 72–81, 1996.

[22] A. Mehta, J. Maggioncalda, O. Bagasra, et al., "In situ DNA PCR and RNA hybridization detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice," *Virology*, vol. 206, no. 1, pp. 633–640, 1995.

[23] R. Ramakrishnan, D. J. Fink, G. Jiang, P. Desai, J. C. Glorioso, and M. Levine, "Competitive quantitative PCR analysis of herpes simplex virus type 1 DNA and latency-associated transcript RNA in latently infected cells of the rat brain," *The Journal of Virology*, vol. 68, no. 3, pp. 1864–1873, 1994.

[24] R. Ramakrishnan, M. Levine, and D. J. Fink, "PCR-based analysis of herpes simplex virus type 1 latency in the rat trigeminal ganglion established with a ribonucleotide reductase-deficient mutant," *The Journal of Virology*, vol. 68, no. 11, pp. 7083–7091, 1994.

[25] N. M. Sawtell, "Comprehensive quantification of herpes simplex virus latency at the single-cell level," *The Journal of Virology*, vol. 71, no. 7, pp. 5423–5431, 1997.

[26] R. F. Itzhaki, W. R. Lin, D. Shang, G. K. Wilcock, B. Faragher, and G. A. Jamieson, "Herpes simplex virus type 1 in brain and risk of Alzheimer's disease," *The Lancet*, vol. 349, no. 9047, pp. 241–244, 1997.

[27] W. Liedtke, B. Opalka, C. W. Zimmermann, and E. Lignitz, "Age distribution of latent herpes simplex virus 1 and varicella-zoster virus genome in human nervous tissue," *Journal of the Neurological Sciences*, vol. 116, no. 1, pp. 6–11, 1993.

[28] P. G. Spear, "Herpes simplex virus: receptors and ligands for cell entry," *Cellular Microbiology*, vol. 6, no. 5, pp. 401–410, 2004.

[29] P. G. Spear, S. Manoj, M. Yoon, C. R. Jogger, A. Zago, and D. Myscofski, "Different receptors binding to distinct interfaces on herpes simplex virus gD can trigger events leading to cell fusion and viral entry," *Virology*, vol. 344, no. 1, pp. 17–24, 2006.

[30] P. G. Spear and R. Longnecker, "Herpesvirus entry: an update," *The Journal of Virology*, vol. 77, no. 19, pp. 10179–10185, 2003.

[31] R. I. Montgomery, M. S. Warner, B. J. Lum, and P. G. Spear, "Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family," *Cell*, vol. 87, no. 3, pp. 427–436, 1996.

[32] R. J. Geraghty, C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear, "Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor," *Science*, vol. 280, no. 5369, pp. 1618–1620, 1998.

[33] R. W. Honess and B. Roizman, "Regulation of herpesvirus macromolecular synthesis. I: cascade regulation of the synthesis of three groups of viral proteins," *The Journal of Virology*, vol. 14, no. 1, pp. 8–19, 1974.

[34] P. O'Hare, "The virion transactivator of herpes simplex virus," *Seminars in Virology*, vol. 4, no. 3, pp. 145–155, 1993.

[35] M. J. Carrozza and N. A. DeLuca, "Interaction of the viral activator protein ICP4 with TFIID through TAF250," *Molecular and Cellular Biology*, vol. 16, no. 6, pp. 3085–3093, 1996.

[36] N. A. DeLuca, A. M. McCarthy, and P. A. Schaffer, "Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4," *The Journal of Virology*, vol. 56, no. 2, pp. 558–570, 1985.

[37] N. A. DeLuca and P. A. Schaffer, "Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4," *Molecular and Cellular Biology*, vol. 5, no. 8, pp. 1997–2008, 1985.

[38] R. A. F. Dixon and P. A. Schaffer, "Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175," *The Journal of Virology*, vol. 36, no. 1, pp. 189–203, 1980.

[39] A. M. McCarthy, L. McMahan, and P. A. Schaffer, "Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient," *The Journal of Virology*, vol. 63, no. 1, pp. 18–27, 1989.

[40] L. McMahan and P. A. Schaffer, "The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection," *The Journal of Virology*, vol. 64, no. 7, pp. 3471–3485, 1990.

[41] W. R. Sacks, C. C. Greene, D. P. Aschman, and P. A. Schaffer, "Herpes simpex virus type 1 ICP27 is an essential regulatory protein," *The Journal of Virology*, vol. 55, no. 3, pp. 796–805, 1985.

[42] M. S. Roberts, A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward, "Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 ($\alpha$4) promoter and a specific binding site for the IE175 (ICP4) protein," *The Journal of Virology*, vol. 62, no. 11, pp. 4307–4320, 1988.

[43] P. O'Hare and G. S. Hayward, "Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation," *The Journal of Virology*, vol. 56, no. 3, pp. 723–733, 1985.

[44] N. Michael and B. Roizman, "Repression of the herpes simplex virus 1 $\alpha$4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 6, pp. 2286–2290, 1993.

[45] B. Gu, R. Rivera-Gonzalez, C. A. Smith, and N. A. DeLuca, "Herpes simplex virus infected cell polypeptide 4 preferentially represses Sp1-activated over basal transcription from its own promoter," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 20, pp. 9528–9532, 1993.

[46] B. Gu and N. DeLuca, "Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4," *The Journal of Virology*, vol. 68, no. 12, pp. 7953–7965, 1994.

[47] C. A. Smith, P. Bates, R. Rivera-Gonzalez, B. Gu, and N. A. DeLuca, "ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB," *The Journal of Virology*, vol. 67, no. 8, pp. 4676–4687, 1993.

[48] R. M. Sandri-Goldin and G. E. Mendoza, "A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing," *Genes and Development*, vol. 6, no. 5, pp. 848–863, 1992.

[49] R. M. Sandri-Goldin, M. K. Hibbard, and M. A. Hardwicke, "The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing," *The Journal of Virology*, vol. 69, no. 10, pp. 6063–6076, 1995.

[50] M. A. Hardwicke and P. A. Schaffer, "Differential effects of nerve growth factor and dexamethasone on herpes simplex virus type 1 oriL- and oriS-dependent DNA replication in PC12 cells," *The Journal of Virology*, vol. 71, no. 5, pp. 3580–3587, 1997.

[51] W. R. Hardy and R. M. Sandri-Goldin, "Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect," *The Journal of Virology*, vol. 68, no. 12, pp. 7790–7799, 1994.

[52] A. Hill, P. Juovic, I. York, et al., "Herpes simplex virus turns off the TAP to evade host immunity," *Nature*, vol. 375, no. 6530, pp. 411–415, 1995.

[53] K. Goldsmith, W. Chen, D. C. Johnson, and R. L. Hendricks, "Infected cell protein (ICP)47 enhances herpes simplex virus neurovirulence by blocking the $CD8^+$ T cell response," *Journal of Experimental Medicine*, vol. 187, no. 3, pp. 341–348, 1998.

[54] R. Jordan and P. A. Schaffer, "Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis," *The Journal of Virology*, vol. 71, no. 9, pp. 6850–6862, 1997.

[55] Y. Kawaguchi, R. Bruni, and B. Roizman, "Interaction of herpes simplex virus 1 $\alpha$1; regulatory protein ICP0 with elongation factor 1$\delta$: ICP0 affects translational machinery," *The Journal of Virology*, vol. 71, no. 2, pp. 1019–1024, 1997.

[56] Y. Kawaguchi, C. Van Sant, and B. Roizman, "Herpes simplex virus 1 $\alpha$ regulatory protein ICP0 interacts with and stabilizes the cell cycle regulator cyclin D3," *The Journal of Virology*, vol. 71, no. 10, pp. 7328–7336, 1997.

[57] M. Meredith, A. Orr, and R. Everett, "Herpes simplex virus type 1 immediate-early protein Vmw110 binds strongly and specifically to a 135-kDa cellular protein," *Virology*, vol. 200, no. 2, pp. 457–469, 1994.

[58] M. Meredith, A. Orr, M. Elliott, and R. Everett, "Separation of sequence requirements for HSV-1 Vmw110 multimerisation and interaction with a 135-kDa cellular protein," *Virology*, vol. 209, no. 1, pp. 174–187, 1995.

[59] R. Everett, P. O'Hare, D. O'Rourke, P. Barlow, and A. Orr, "Point mutations in the herpes simplex virus type 1 Vmw110 RING finger helix affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures," *The Journal of Virology*, vol. 69, no. 11, pp. 7339–7344, 1995.

[60] R. D. Everett, P. Lomonte, T. Sternsdorf, R. van Driel, and A. Orr, "Cell cycle regulation of PML modification and ND10 composition," *Journal of Cell Science*, vol. 112, no. 24, pp. 4581–4588, 1999.

[61] R. D. Everett, M. Meredith, A. Orr, A. Cross, M. Kathoria, and J. Parkinson, "A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein," *The EMBO Journal*, vol. 16, no. 3, pp. 566–577, 1997.

[62] R. D. Everett, M. Meredith, A. Orr, A. Cross, M. Kathoria, and J. Parkinson, "A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein," *The EMBO Journal*, vol. 16, no. 7, pp. 1519–1530, 1997.

[63] A. P. W. Poon, Y. Liang, and B. Roizman, "Herpes simplex virus 1 gene expression is accelerated by inhibitors of histone deacetylases in rabbit skin cellls infected with a mutant carrying a cDNA copy of the infected cell protein no. 0," *The Journal of Virology*, vol. 77, no. 23, pp. 12671–12678, 2003.

[64] W. E. Hobbs II and N. A. DeLuca, "Perturbation of cell cycle progression and cellular gene expression as a function of herpes simplex virus ICP0," *The Journal of Virology*, vol. 73, no. 10, pp. 8245–8255, 1999.

[65] H. Gu, Y. Liang, G. Mandel, and B. Roizman, "Components of the REST/CoREST/histone deacytlase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, pp. 7571–7576, 2005.

[66] P. Lomonte, D. Seigneurin-Berny, R. D. Everett, S. Khochbin, and A. L. Epstein, "Interaction of HSV-1 ICP0 with class II histone deacytlases," in *Proceedings of the 26th International Herpesvirus Workshop*, Regensburg, Germany, 2001.

[67] A. P. W. Poon, H. Gu, and B. Roizman, "ICP0 and the US3 protein kinase of herpes simplex virus 1 independently block histone deacetylation to enable gene expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 26, pp. 9993–9998, 2006.

[68] A. R. Cliffe and D. M. Knipe, "Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection," *The Journal of Virology*, vol. 82, no. 24, pp. 12030–12038, 2008.

[69] W. Cai and P. A. Schaffer, "A cellular function can enhance gene expression and plating efficiency of a mutant defective in the gene for ICP0, a transactivating protein of herpes simplex virus type 1," *The Journal of Virology*, vol. 65, no. 8, pp. 4078–4090, 1991.

[70] F. B. Knotts, M. L. Cook, and J. G. Stevens, "Pathogenesis of herpetic encephalitis in mice after ophthalmic inoculation," *Journal of Infectious Diseases*, vol. 130, no. 1, pp. 16–27, 1974.

[71] M. F. Kramer, S. H. Chen, D. M. Knipe, and D. M. Coen, "Accumulation of viral transcripts and DNA during establishment of latency by herpes simplex virus," *The Journal of Virology*, vol. 72, no. 2, pp. 1177–1185, 1998.

[72] P. G. Speck and A. Simmons, "Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1," *The Journal of Virology*, vol. 65, no. 8, pp. 4001–4005, 1991.

[73] D. M. Coen, M. Kosz-Vnenchak, J. G. Jacobson, et al., "Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 12, pp. 4736–4740, 1989.

[74] D. A. Leib, D. M. Coen, C. L. Bogard, et al., "Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency," *The Journal of Virology*, vol. 63, no. 2, pp. 759–768, 1989.

[75] S. Efstathiou, S. Kemp, G. Darby, and A. C. Minson, "The role of herpes simplex virus type 1 thymidine kinase in pathogenesis," *Journal of General Virology*, vol. 70, no. 4, pp. 869–879, 1989.

[76] J. P. Katz, E. T. Bodin, and D. M. Coen, "Quantitative polymerase chain reaction analysis of herpes simplex virus DNA in ganglia of mice infected with replication-incompetent mutants," *The Journal of Virology*, vol. 64, no. 9, pp. 4288–4295, 1990.

[77] T. P. Margolis, F. Sedarati, A. T. Dobson, L. T. Feldman, and J. G. Stevens, "Pathways of viral gene expression during acute neuronal infection with HSV-1," *Virology*, vol. 189, no. 1, pp. 150–160, 1992.

[78] F. Sedarati, K. M. Izumi, E. K. Wagner, and J. G. Stevens, "Herpes simplex virus type 1 latency-associated transcription plays no role in establishment or maintenance of a latent infection in murine sensory neurons," *The Journal of Virology*, vol. 63, no. 10, pp. 4455–4458, 1989.

[79] F. Sedarati, T. P. Margolis, and J. G. Stevens, "Latent infection can be established with drastically restricted transcription and replication of the HSV-1 genome," *Virology*, vol. 192, no. 2, pp. 687–691, 1993.

[80] I. Steiner, J. G. Spivack, S. L. Deshmane, C. I. Ace, C. M. Preston, and N. W. Fraser, "A herpes simplex virus type 1 mutant containing a nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia," *The Journal of Virology*, vol. 64, no. 4, pp. 1630–1638, 1990.

[81] T. Valyi-Nagy, S. L. Deshmane, B. Raengsakulrach, et al., "Herpes simplex virus type 1 mutant strain in1814 establishes a unique, slowly progressing infection in SCID mice," *The Journal of Virology*, vol. 66, no. 12, pp. 7336–7345, 1992.

[82] N. S. Taus and W. J. Mitchell, "The transgenic ICP4 promoter is activated in Schwann cells in trigeminal ganglia of mice latently infected with herpes simplex virus type 1," *The Journal of Virology*, vol. 75, no. 21, pp. 10401–10408, 2001.

[83] C. M. Loiacono, R. Myers, and W. J. Mitchell, "Neurons differentially activate the herpes simplex virus type 1 immediate-early gene ICP0 and ICP27 promoters in transgenic mice," *The Journal of Virology*, vol. 76, no. 5, pp. 2449–2459, 2002.

[84] L. R. Devireddy and C. J. Jones, "Olf-1, a neuron-specific transcription factor, can activate the herpes simplex virus type 1-infected cell protein 0 promoter," *The Journal of Biological Chemistry*, vol. 275, no. 1, pp. 77–81, 2000.

[85] P. O'Hare and C. R. Goding, "Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation," *Cell*, vol. 52, no. 3, pp. 435–445, 1988.

[86] R. Lu and V. Misra, "Zhangfei: a second cellular protein interacts with herpes simplex virus accessory factor HCF in a manner similar to Luman and VP16," *Nucleic Acids Research*, vol. 28, no. 12, pp. 2446–2454, 2000.

[87] T. M. Kristie, J. L. Pomerantz, T. C. Twomey, S. A. Parent, and P. A. Sharp, "The cellular C1 factor of the herpes simplex virus enhancer complex is a family of polypeptides," *The Journal of Biological Chemistry*, vol. 270, no. 9, pp. 4387–4394, 1995.

[88] T. M. Kristie, J. L. Vogel, and A. E. Sears, "Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 4, pp. 1229–1233, 1999.

[89] A. E. Sears, V. Hukkanen, M. A. Labow, A. J. Levine, and B. Roizman, "Expression of the herpes simplex virus 1 $\alpha$ transinducing factor (VP16) does not induce reactivation of latent virus or prevent the establishment of latency in mice," *The Journal of Virology*, vol. 65, no. 6, pp. 2929–2935, 1991.

[90] M. Hagmann, O. Georgiev, W. Schaffner, and P. Douville, "Transcription factors interacting with herpes simplex virus $\alpha$ gene promoters in sensory neurons," *Nucleic Acids Research*, vol. 23, no. 24, pp. 4978–4985, 1995.

[91] E. E. Turner, J. M. Rhee, and L. T. Feldman, "The POU-domain factor Brn-3.0 recognizes characteristic sites in the herpes simplex virus genome," *Nucleic Acids Research*, vol. 25, no. 13, pp. 2589–2594, 1997.

[92] C. A. Gruber, J. M. Rhee, A. Gleiberman, and E. E. Turner, "POU domain factors of the Brn-3 class recognize functional DNA elements which are distinctive, symmetrical, and highly conserved in evolution," *Molecular and Cellular Biology*, vol. 17, no. 5, pp. 2391–2400, 1997.

[93] R. J. McEvilly, L. Erkman, L. Luo, P. E. Sawchenko, A. F. Ryan, and M. G. Rosenfeld, "Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons," *Nature*, vol. 384, no. 6609, pp. 574–577, 1996.

[94] M. D. Schonemann, A. K. Ryan, L. Erkman, R. J. McEvilly, J. Bermingham, and M. G. Rosenfeld, "POU domain factors in neural development," *Advances in Experimental Medicine and Biology*, vol. 449, pp. 39–53, 1998.

[95] L. Erkman, R. J. McEvilly, L. Luo, et al., "Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development," *Nature*, vol. 381, no. 6583, pp. 603–606, 1996.

[96] P. J. Morris, T. Theil, C. J. A. Ring, K. A. Lillycrop, T. Moroy, and D. S. Latchman, "The opposite and antagonistic effects of the closely related POU family transcription factors Brn-3a and Brn-3b on the activity of a target promoter are dependent on differences in the POU domain," *Molecular and Cellular Biology*, vol. 14, no. 10, pp. 6907–6914, 1994.

[97] X.-P. Chen, J. Li, M. Mata, et al., "Herpes simplex virus type 1 ICP0 protein does not accumulate in the nucleus of primary neurons in culture," *The Journal of Virology*, vol. 74, no. 21, pp. 10132–10141, 2000.

[98] W. L. Hsu and R. D. Everett, "Human neuron-committed teratocarcinoma NT2 cell line has abnormal ND10 structures and is poorly infected by herpes simplex virus type 1," *The Journal of Virology*, vol. 75, no. 8, pp. 3819–3831, 2001.

[99] X. Chen, M. C. Schmidt, W. F. Goins, and J. C. Glorioso, "Two herpes simplex virus type 1 latency-active promoters differ in their contributions to latency-associated transcript expression during lytic and latent infections," *The Journal of Virology*, vol. 69, no. 12, pp. 7899–7908, 1995.

[100] W. F. Goins, L. R. Sternberg, K. D. Croen, et al., "A novel latency-active promoter is contained within the herpes simplex virus type 1 U(L) flanking repeats," *The Journal of Virology*, vol. 68, no. 4, pp. 2239–2252, 1994.

[101] S. L. Deshmane, M. Nicosia, T. Valyi-Nagy, L. T. Feldman, A. Dillner, and N. W. Fraser, "An HSV-1 mutant lacking the LAT TATA element reactivates normally in explant cocultivation," *Virology*, vol. 196, no. 2, pp. 868–872, 1993.

[102] A. T. Dobson, T. P. Margolis, W. A. Gomes, and L. T. Feldman, "In vivo deletion analysis of the herpes simplex virus type 1 latency- associated transcript promoter," *The Journal of Virology*, vol. 69, no. 4, pp. 2264–2270, 1995.

[103] A. T. Dobson, F. Sederati, G. Devi-Rao, et al., "Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus," *The Journal of Virology*, vol. 63, no. 9, pp. 3844–3851, 1989.

[104] W. J. Mitchell, R. P. Lirette, and N. W. Fraser, "Mapping of low abundance latency-associated RNA in the trigeminal ganglia of mice latently infected with herpes simplex virus type 1," *Journal of General Virology*, vol. 71, no. 1, pp. 125–132, 1990.

[105] A. H. Batchelor and P. O'Hare, "Regulation and cell-type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1," *The Journal of Virology*, vol. 64, no. 7, pp. 3269–3279, 1990.

[106] A. H. Batchelor and P. O'Hare, "Localization of cis-acting sequence requirements in the promoter of the latency-associated transcript of herpes simplex virus type 1 required for cell-type-specific activity," *The Journal of Virology*, vol. 66, no. 6, pp. 3573–3582, 1992.

[107] A. H. Batchelor, K. W. Wilcox, and P. O'Hare, "Binding and repression of the latency-associated promoter of herpes simplex virus by the immediate early 175K protein," *Journal of General Virology*, vol. 75, no. 4, pp. 753–767, 1994.

[108] J. C. Zwaagstra, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler, "Identification of a major regulatory sequence in the latency associated transcript (LAT) promoter of herpes simplex virus type 1 (HSV-1)," *Virology*, vol. 182, no. 1, pp. 287–297, 1991.

[109] J. C. Zwaagstra, H. Ghiasi, S. M. Slanina, et al., "Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript," *The Journal of Virology*, vol. 64, no. 10, pp. 5019–5028, 1990.

[110] J. J. Kenny, F. C. Krebs, H. T. Hartle, et al., "Identification of a second ATF/CREB-like element in the herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) promoter," *Virology*, vol. 200, no. 1, pp. 220–235, 1994.

[111] D. A. Leib, K. C. Nadeau, S. A. Rundle, and P. A. Schaffer, "The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: role of the latency-associated transcripts and cAMP in reactivation of viral latency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 1, pp. 48–52, 1991.

[112] D. C. Bloom, J. G. Stevens, J. M. Hill, and R. K. Tran, "Mutagenesis of a cAMP response element within the latency-associated transcript promoter of HSV-1 reduces adrenergic reactivation," *Virology*, vol. 236, no. 1, pp. 202–207, 1997.

[113] K. A. Rader, C.E. Ackland-Berglund, J. K. Miller, J. S. Pepose, and D. A. Leib, "In vivo characterization of site-directed mutations in the promoter of the herpes simplex virus type 1 latency-associated transcripts," *Journal of General Virology*, vol. 74, no. 9, pp. 1859–1869, 1993.

[114] D. L. Rock, A. B. Nesburn, H. Ghiasi, et al., "Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1," *The Journal of Virology*, vol. 61, no. 12, pp. 3820–3826, 1987.

[115] J. G. Stevens, E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman, "RNA complementary to a herpesvirus $\alpha$ gene mRNA is prominent in latently infected neurons," *Science*, vol. 235, no. 4792, pp. 1056–1059, 1987.

[116] J. Chou, E. R. Kern, R. J. Whitley, and B. Roizman, "Mapping of herpes simplex virus-1 neurovirulence to $\gamma$134.5, a gene nonessential for growth in culture," *Science*, vol. 250, no. 4985, pp. 1262–1266, 1990.

[117] J. Chou and B. Roizman, "The herpes simplex virus 1 gene for ICP34.5, which maps in inverted repeats, is conserved in several limited-passage isolates but not in strain 17syn+," *Journal of Virology*, vol. 64, no. 3, pp. 1014–1020, 1990.

[118] M. Lagunoff and B. Roizman, "Expression of a herpes simplex virus 1 open reading frame antisense to the $\gamma(1)$34.5 gene and transcribed by an RNA 3′ coterminal with the unspliced latency-associated transcript," *Journal of Virology*, vol. 68, no. 9, pp. 6021–6028, 1994.

[119] L. Yeh and P. A. Schaffer, "A novel class of transcripts expressed with late kinetics in the absence of ICP4 spans the junction between the long and short segments of the herpes simplex virus type 1 genome," *Journal of Virology*, vol. 67, no. 12, pp. 7373–7382, 1993.

[120] R. A. Bohenzky, M. Lagunoff, B. Roizman, E. K. Wagner, and S. Silverstein, "Two overlapping transcription units which extend across the L-S junction of herpes simplex virus type 1," *Journal of Virology*, vol. 69, no. 5, pp. 2889–2897, 1995.

[121] R. A. Bohenzky, A. G. Papavassiliou, I. H. Gelman, and S. Silverstein, "Identification of a promoter mapping within the reiterated sequences that flank the herpes simplex virus type 1 UL region," *Journal of Virology*, vol. 67, no. 2, pp. 632–642, 1993.

[122] H. Berthomme, J. Lokensgard, L. Yang, T. Margolis, and L. T. Feldman, "Evidence for a bidirectional element located downstream from the herpes simplex virus type 1 latency-associated promoter that increases its activity during latency," *The Journal of Virology*, vol. 74, no. 8, pp. 3613–3622, 2000.

[123] J. R. Lokensgard, H. Berthomme, and L. T. Feldman, "The latency-associated promoter of herpes simplex virus type 1 requires a region downstream of the transcription start site for long-term expression during latency," *The Journal of Virology*, vol. 71, no. 9, pp. 6714–6719, 1997.

[124] J. R. Lokensgard, H. Berthomme, and L. T. Feldman, "The latency-associated promoter of herpes simplex virus type 1 requires a region downstream of the transcription start site for long-term expression during latency," *The Journal of Virology*, vol. 71, no. 9, pp. 6714–6719, 1997.

[125] J. R. Lokensgard, D. C. Bloom, A. T. Dobson, and L. T. Feldman, "Long-term promoter activity during herpes simplex virus latency," *The Journal of Virology*, vol. 68, no. 11, pp. 7148–7158, 1994.

[126] N. J. Kubat, R. K. Tran, P. McAnany, and D. C. Bloom, "Specific histone tail modification and not DNA methylation is a determinant of herpes simplex virus type 1 latent gene expression," *The Journal of Virology*, vol. 78, no. 3, pp. 1139–1149, 2004.

[127] A. M. Deatly, J. G. Spivack, E. Lavi, and N. W. Fraser, "RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 10, pp. 3204–3208, 1987.

[128] A. M. Deatly, J. G. Spivack, E. Lavi, D. R. O'Boyle II, and N. W. Fraser, "Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome," *The Journal of Virology*, vol. 62, no. 3, pp. 749–756, 1988.

[129] P. R. Krause, K. D. Croen, S. E. Straus, and J. M. Ostrove, "Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia," *The Journal of Virology*, vol. 62, no. 12, pp. 4819–4823, 1988.

[130] E. K. Wagner, W. M. Flanagan, G. Devi-Rao, et al., "The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection," *The Journal of Virology*, vol. 62, no. 12, pp. 4577–4585, 1988.

[131] E. K. Wagner, G. Devi-Rao, L. T. Feldman, et al., "Physical characterization of the herpes simplex virus latency-associated transcript in neurons," *The Journal of Virology*, vol. 62, no. 4, pp. 1194–1202, 1988.

[132] G. C. Perng, H. Ghiasi, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler, "The spontaneous reactivation function of the herpes simplex virus type 1 LAT gene resides completely within the first 1.5 kilobases of the 8.3-kilobase primary transcript," *The Journal of Virology*, vol. 70, no. 2, pp. 976–984, 1996.

[133] M. F. Kramer and D. M. Coen, "Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus," *The Journal of Virology*, vol. 69, no. 3, pp. 1389–1399, 1995.

[134] C. Shimeld, T. J. Hill, W. A. Blyth, and D. L. Easty, "Reactivation of latent infection and induction of recurrent herpetic eye disease in mice," *Journal of General Virology*, vol. 71, no. 2, pp. 397–404, 1990.

[135] M. T. Green, R. J. Courtney, and E. C. Dunkel, "Detection of an immediate early herpes simplex virus type 1 polypeptide in trigeminal ganglia from latently infected animals," *Infection and Immunity*, vol. 34, no. 3, pp. 987–992, 1981.

[136] J. L. Umbach, M. F. Kramer, I. Jurak, H. W. Karnowski, D. M. Coen, and B. R. Cullen, "MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs," *Nature*, vol. 454, no. 7205, pp. 780–783, 2008.

[137] B. S. Drolet, G.-C. Perng, J. Cohen, et al., "The region of the herpes simplex virus type 1 LAT gene involved in spontaneous reactivation does not encode a functional protein," *Virology*, vol. 242, no. 1, pp. 221–232, 1998.

[138] W. Peng, O. Vitvitskaia, D. Carpenter, S. L. Wechsler, and C. Jones, "Identification of two small RNAs within the first 1.5-kb of the herpes simplex virus type 1-encoded latency-associated transcript," *Journal of NeuroVirology*, vol. 14, no. 1, pp. 41–52, 2008.

[139] W. Kang, R. Mukerjee, and N. F. Fraser, "Establishment and maintenance of HSV latent infection is mediated through correct splicing of the LAT primary transcript," *Virology*, vol. 312, no. 1, pp. 233–244, 2003.

[140] N. Mador, A. Panet, D. Latchman, and I. Steiner, "Expression and splicing of the latency-associated transcripts of herpes simplex virus type 1 in neuronal and non-neuronal cell lines," *Journal of Biochemistry*, vol. 117, no. 6, pp. 1288–1297, 1995.

[141] C. Krummenacher, J. M. Zabolotny, and N. W. Fraser, "Selection of a nonconsensus branch point is influenced by an RNA stem-loop structure and is important to confer stability to the herpes simplex virus 2-kilobase latency-associated transcript," *The Journal of Virology*, vol. 71, no. 8, pp. 5849–5860, 1997.

[142] M. J. Farrell, A. T. Dobson, and L. T. Feldman, "Herpes simplex virus latency-associated transcript is a stable intron," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 3, pp. 790–794, 1991.

[143] M. Ahmed and N. W. Fraser, "Herpes simplex virus type 1 2-kilobase latency-associated transcript intron associates with ribosomal proteins and splicing factors," *The Journal of Virology*, vol. 75, no. 24, pp. 12070–12080, 2001.

[144] M. Nicosia, J. M. Zabolotny, R. P. Lirette, and N. W. Fraser, "The HSV-1 2-kb latency-associated transcript is found in the cytoplasm comigrating with ribosomal subunits during productive infection," *Virology*, vol. 204, no. 2, pp. 717–728, 1994.

[145] D. L. Thomas, M. Lock, J. M. Zabolotny, B. R. Mohan, and N. W. Fraser, "The 2-kilobase intron of the herpes simplex virus type 1 latency-associated transcript has a half-life of approximately 24 hours in SY5Y and COS-1 cells," *Journal of Virology*, vol. 76, no. 2, pp. 532–540, 2002.

[146] D. Goldenberg, N. Mador, M. J. Ball, A. Panet, and I. Steiner, "The abundant latency-associated transcripts of herpes simplex virus type 1 are bound to polyribosomes in cultured neuronal cells and during latent infection in mouse trigeminal ganglia," *Journal of Virology*, vol. 71, no. 4, pp. 2897–2904, 1997.

[147] G. J. Hannon, "RNA interference," *Nature*, vol. 418, no. 6894, pp. 244–251, 2002.

[148] D. M. Dykxhoorn, C. D. Novina, and P. A. Sharp, "Killing the messenger: short RNAs that silence gene expression," *Nature Reviews Molecular Cell Biology*, vol. 4, no. 6, pp. 457–467, 2003.

[149] T. Kuwabara, J. Hsieh, K. Nakashima, K. Taira, and F. H. Gage, "A small modulatory dsRNA specifies the fate of adult neural stem cells," *Cell*, vol. 116, no. 6, pp. 779–793, 2004.

[150] P. Xu, M. Guo, and B. A. Hay, "microRNAs and the regulation of cell death," *Trends in Genetics*, vol. 20, no. 12, pp. 617–624, 2004.

[151] E. J. Finnegan and M. A. Matzke, "The small RNA world," *Journal of Cell Science*, vol. 116, no. 23, pp. 4689–4693, 2003.

[152] D. Banerjee and F. Slack, "Control of developmental timing by small temporal rnas: a paradigm for rna-mediated regulation of gene expression," *BioEssays*, vol. 24, no. 2, pp. 119–129, 2002.

[153] B. J. Reinhart and D. P. Bartel, "Small RNAs correspond to centromere heterochromatic repeats," *Science*, vol. 297, no. 5588, pp. 1831–1835, 2002.

[154] A. Fraser, "Worms in LA," *Nature Genetics*, vol. 35, pp. 3–5, 2003.

[155] D. P. Bartel, "microRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[156] S. Pfeffer, M. Zavolan, F. A. Grasser, et al., "Identification of virus-encoded microRNAs," *Science*, vol. 304, no. 5671, pp. 734–736, 2004.

[157] S. Pfeffer, A. Sewer, M. Lagos-Quintana, et al., "Identification of microRNAs of the herpesvirus family," *Nature Methods*, vol. 2, no. 4, pp. 269–276, 2005.

[158] X. Cai, S. Lu, Z. Zhang, C. M. Gonzalez, B. Damania, and B. R. Cullen, "Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 15, pp. 5570–5575, 2005.

[159] M. A. Samols, J. Hu, R. L. Skalsky, and R. Renne, "Cloning and identification of a microRNA cluster within the latency-associated region of Kapos's sarcoma-associated herpesvirus," *Journal of Virology*, vol. 79, no. 14, pp. 9301–9305, 2005.

[160] F. Grey, A. Antoniewicz, E. Allen, et al., "Identification and characterization of human cytomegalovirus-encoded microRNAs," *Journal of Virology*, vol. 79, no. 18, pp. 12095–12099, 2005.

[161] W. Dunn, P. Trang, Q. Zhong, E. Yang, C. van Belle, and F. Liu, "Human cytomegalovirus expresses novel microRNAs during productive viral infection," *Cellular Microbiology*, vol. 7, no. 11, pp. 1684–1695, 2005.

[162] A. Gupta, J. J. Gartner, P. Sethupathy, A. G. Hatzigeorgiou, and N. W. Fraser, "Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript," *Nature*, vol. 442, no. 7098, pp. 82–85, 2006.

[163] J. Burnside, E. Bernberg, A. Anderson, et al., "Marek's disease virus encodes microRNAs that map to meq and the latency-associated transcript," *Journal of Virology*, vol. 80, no. 17, pp. 8778–8786, 2006.

[164] C. S. Sullivan, A. T. Grundhoff, S. Tevethia, J. M. Pipas, and D. Ganem, "SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells," *Nature*, vol. 435, no. 7042, pp. 682–686, 2005.

[165] W. Shen, M. S. Silva, T. Jaber, et al., "Two small RNAs encoded within the first 1.5 kilobases of the herpes simplex virus type 1 latency-associated transcript can inhibit productive infection and cooperate to inhibit apoptosis," *Journal of Virology*, vol. 83, no. 18, pp. 9131–9139, 2009.

[166] J. Singh and E. Wagner, "Transcriptional analysis of the herpes simplex virus type 1 region containing the TRL/UL junction," *Virology*, vol. 196, no. 1, pp. 220–231, 1993.

[167] J. Naito, R. Mukerjee, K. R. Mott, et al., "Identification of a protein encoded in the herpes simplex virus type 1 latency associated transcript promoter region," *Virus Research*, vol. 108, no. 1-2, pp. 101–110, 2005.

[168] D. Chan, J. Cohen, J. Naito, et al., "A mutant deleted for most of the herpes simplex virus type 1 (HSV-1) UOL gene does not affect the spontaneous reactivation phenotype in rabbits," *Journal of NeuroVirology*, vol. 12, no. 1, pp. 5–16, 2006.

[169] G.-C. Perng, B. Maguen, L. Jin, et al., "A novel herpes simplex virus type 1 transcript (AL-RNA) antisense to the 5′ end of the latency-associated transcript produces a protein in infected rabbits," *Journal of Virology*, vol. 76, no. 16, pp. 8003–8010, 2002.

[170] T. Jaber, G. Henderson, S. Li, et al., "Identification of a novel herpes simplex virus type 1 transcript and protein (AL3) expressed during latency," *Journal of General Virology*, vol. 90, no. 10, pp. 2342–2352, 2009.

[171] C. Jones, "Herpes simplex virus type 1 and bovine herpesvirus 1 latency," *Clinical Microbiology Reviews*, vol. 16, no. 1, pp. 79–95, 2003.

[172] T. M. Block, J. G. Spivack, I. Steiner, et al., "A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection," *Journal of Virology*, vol. 64, no. 7, pp. 3417–3426, 1990.

[173] D. Y. Ho and E. S. Mocarski, "$\beta$-galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse," *Virology*, vol. 167, no. 1, pp. 279–283, 1988.

[174] R. L. Thompson and N. M. Sawtell, "The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency," *Journal of Virology*, vol. 71, no. 7, pp. 5432–5440, 1997.

[175] N. M. Sawtell and R. L. Thompson, "Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency," *Journal of Virology*, vol. 66, no. 4, pp. 2157–2169, 1992.

[176] G. B. Devi-Rao, D. C. Bloom, J. G. Stevens, and E. K. Wagner, "Herpes simplex virus type 1 DNA replication and gene expression during explant-induced reactivation of latently infected murine sensory ganglia," *Journal of Virology*, vol. 68, no. 3, pp. 1271–1282, 1994.

[177] G.-C. Perng, H. Ghiasi, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler, "The spontaneous reactivation function of the herpes simplex virus type 1 LAT gene resides completely within the first 1.5 kilobases of the 8.3-kilobase primary transcript," *Journal of Virology*, vol. 70, no. 2, pp. 976–984, 1996.

[178] S.-H. Chen, M. F. Kramer, P. A. Schaffer, and D. M. Coen, "A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus," *Journal of Virology*, vol. 71, no. 8, pp. 5878–5884, 1997.

[179] D. A. Garber, P. A. Schaffer, and D. M. Knipe, "A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1," *Journal of Virology*, vol. 71, no. 8, pp. 5885–5893, 1997.

[180] G.-C. Perng, S. M. Slanina, A. Yukht, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler, "The latency-associated transcript gene enhances establishment of herpes simplex virus type 1 latency in rabbits," *Journal of Virology*, vol. 74, no. 4, pp. 1885–1891, 2000.

[181] G.-C. Perng, E. C. Dunkel, P. A. Geary, et al., "The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency," *Journal of Virology*, vol. 68, no. 12, pp. 8045–8055, 1994.

[182] G.-C. Perng, K. Chokephaibulkit, R. L. Thompson, et al., "The region of the herpes simplex virus type 1 LAT gene that is colinear with the ICP34.5 gene is not involved in spontaneous reactivation," *Journal of Virology*, vol. 70, no. 1, pp. 282–291, 1996.

[183] G.-C. Perng, S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler, "A 371-nucleotide region between the herpes simplex virus type 1 (HSV-1) LAT promoter and the 2-kilobase LAT is not essential for efficient spontaneous reactivation of latent HSV-1," *Journal of Virology*, vol. 70, no. 3, pp. 2014–2018, 1996.

[184] G.-C. Perng, S. M. Slanina, A. Yukht, et al., "A herpes simplex virus type 1 latency-associated transcript mutant with increased virulence and reduced spontaneous reactivation," *Journal of Virology*, vol. 73, no. 2, pp. 920–929, 1999.

[185] M. D. Trousdale, I. Steiner, J. G. Spivack, et al., "In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model," *Journal of Virology*, vol. 65, no. 12, pp. 6989–6993, 1991.

[186] J. M. Hill, F. Sedarati, R. T. Javier, E. K. Wagner, and J. G. Stevens, "Herpes simplex virus latent phase transcription facilitates in vivo reactivation," *Virology*, vol. 174, no. 1, pp. 117–125, 1990.

[187] G.-C. Perng, S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler, "The effect of latency-associated transcript on the herpes simplex virus type 1 latency-reactivation phenotype is mouse strain-dependent," *Journal of General Virology*, vol. 82, no. 5, pp. 1117–1122, 2001.

[188] C. Doerig, L. I. Pizer, and C. L. Wilcox, "An antigen encoded by the latency-associated transcript in neuronal cell cultures latently infected with herpes simplex virus type 1," *Journal of Virology*, vol. 65, no. 5, pp. 2724–2727, 1991.

[189] M. Lock, C. Miller, and N. W. Fraser, "Analysis of protein expression from within the region encoding the 2.0-kilobase latency-associated transcript of herpes simplex virus type 1," *Journal of Virology*, vol. 75, no. 7, pp. 3413–3426, 2001.

[190] G. Randall, M. Lagunoff, and B. Roizman, "The product of ORF O located within the domain of herpes simplex virus 1 genome transcribed during latent infection binds to and inhibits in vitro binding of infected cell protein 4 to its cognate DNA site," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 19, pp. 10379–10384, 1997.

[191] S. K. Thomas, G. Gough, D. S. Latchman, and R. S. Coffin, "Herpes simplex virus latency-associated transcript encodes a protein which greatly enhances virus growth, can compensate for deficiencies in immediate-early gene expression, and is likely to function during reactivation from virus latency," *Journal of Virology*, vol. 73, no. 8, pp. 6618–6625, 1999.

[192] S. K. Thomas, C. E. Lilley, D. S. Latchman, and R. S. Coffin, "A protein encoded by the herpes simplex virus (HSV) type 1 2-kilobase latency-associated transcript is phosphorylated, localized to the nucleus, and overcomes the repression of expression from exogenous promoters when inserted into the quiescent HSV genome," *Journal of Virology*, vol. 76, no. 8, pp. 4056–4067, 2002.

[193] G.-C. Perng, D. Esmaili, S. M. Slanina, et al., "Three herpes simplex virus type 1 latency-associated transcript mutants with distinct and asymmetric effects on virulence in mice compared with rabbits," *Journal of Virology*, vol. 75, no. 19, pp. 9018–9028, 2001.

[194] G. Henderson, T. Jaber, D. Carpenter, S. L. Wechsler, and C. Jones, "Identification of herpes simplex virus type 1 (HSV-1) proteins encoded within the first 1.5 kb of the latency-associated transcript (LAT)," *Journal of Neurovirology*. In press.

[195] J. M. Hardwick, "Viral interference with apoptosis," *Seminars in Cell and Developmental Biology*, vol. 9, no. 3, pp. 339–349, 1998.

[196] E. S. Razvi and R. M. Welsh, "Apoptosis in viral infections," *Advances in Virus Research*, vol. 45, pp. 1–60, 1995.

[197] Y. Shen and T. E. Shenk, "Viruses and apoptosis," *Current Opinion in Genetics and Development*, vol. 5, no. 1, pp. 105–111, 1995.

[198] J. G. Teodoro and P. E. Branton, "Regulation of apoptosis by viral gene products," *Journal of Virology*, vol. 71, no. 3, pp. 1739–1746, 1997.

[199] L. R. Devireddy and C. J. Jones, "Activation of caspases and p53 by bovine herpesvirus 1 infection results in programmed cell death and efficient virus release," *Journal of Virology*, vol. 73, no. 5, pp. 3778–3788, 1999.

[200] V. Galvan, R. Brandimarti, and B. Roizman, "Herpes simplex virus 1 blocks caspase-3-independent and caspase-dependent pathways to cell death," *Journal of Virology*, vol. 73, no. 4, pp. 3219–3226, 1999.

[201] V. Galvan and B. Roizman, "Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3931–3936, 1998.

[202] C. Sadzot-Delvaux, P. Thonard, S. Schoonbroodt, J. Piette, and B. Rentier, "Varicella-zoster virus induces apoptosis in cell culture," *Journal of General Virology*, vol. 76, no. 11, pp. 2875–2879, 1995.

[203] S. Asano, T. Honda, F. Goshima, et al., "US3 protein kinase of herpes simplex virus type 2 plays a role in protecting corneal epithelial cells from apoptosis in infected mice," *Journal of General Virology*, vol. 80, no. 1, pp. 51–56, 1999.

[204] M. Aubert and J. A. Blaho, "The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells," *Journal of Virology*, vol. 73, no. 4, pp. 2803–2813, 1999.

[205] R. Leopardi and B. Roizman, "The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 18, pp. 9583–9587, 1996.

[206] M. Ahmed and N. W. Fraser, "Herpes simplex virus type 1 2-kilobase latency-associated transcript intron associates with ribosomal proteins and splicing factors," *Journal of Virology*, vol. 75, no. 24, pp. 12070–12080, 2001.

[207] J. A. Blaho and M. Aubert, "Modulation of apoptosis during herpes simplex virus infection in human cells," *Microbes and Infection*, vol. 3, pp. 1–8, 2001.

[208] K. R. Jerome, Z. Chen, R. Lang, et al., "HSV and glycoprotein J inhibit caspase activation and apoptosis induced by granzyme B or Fas," *Journal of Immunology*, vol. 167, no. 7, pp. 3928–3935, 2001.

[209] K. R. Jerome, R. Fox, Z. Chen, A. E. Sears, H.-Y. Lee, and L. Corey, "Herpes simplex virus inhibits apoptosis through the action of two genes, Us5 and Us3," *Journal of Virology*, vol. 73, no. 11, pp. 8950–8957, 1999.

[210] Y. Jiang, M. Inman, Y. Zhang, N. A. Posadas, and C. Jones, "A mutation in the latency-related gene of bovine herpesvirus 1 inhibits protein expression from open reading frame 2 and an adjacent reading frame during productive infection," *Journal of Virology*, vol. 78, no. 6, pp. 3184–3189, 2004.

[211] J. Munger, A. V. Chee, and B. Roizman, "The $U_S3$ protein kinase blocks apoptosis induced by the *d*120 mutant of herpes simplex virus 1 at a premitochondrial stage," *Journal of Virology*, vol. 75, no. 12, pp. 5491–5497, 2001.

[212] J. Munger and B. Roizman, "The $U_S3$ protein kinase of herpes simplex virus 1 mediates the posttranslational modification of BAD and prevents BAD-induced programmed cell death in the absence of other viral proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 18, pp. 10410–10415, 2001.

[213] C. Chenet-Monte, F. Mohammad, C. M. Celluzzi, P. A. Schaffer, and F. E. Farber, "Herpes simplex virus gene products involved in the induction of chromosomal aberrations," *Virus Research*, vol. 6, no. 3, pp. 245–260, 1986.

[214] B. H. Ellison and S. A. Ellison, "Chromosomal aberrations induced by an animal virus," *Nature*, vol. 192, no. 4798, pp. 145–147, 1961.

[215] R. Heilbronn and H. zur Hausen, "A subset of herpes simplex virus replication genes induces DNA amplification within the host cell genome," *Journal of Virology*, vol. 63, no. 9, pp. 3683–3692, 1989.

[216] L. Pilon, Y. Langelier, and A. Royal, "Herpes simplex virus type 2 mutagenesis: characterization of mutants induced at the hprt locus of nonpermissive XC cells," *Molecular and Cellular Biology*, vol. 6, no. 8, pp. 2977–2983, 1986.

[217] M. S. Soengas, R. M. Alarcon, H. Yoshida, et al., "Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition," *Science*, vol. 284, no. 5411, pp. 156–159, 1999.

[218] R. Hagglund, J. Munger, A. P. W. Poon, and B. Roizman, "$U_S3$ protein kinase of herpes simplex virus 1 blocks caspase 3 activation induced by the products of $U_S1.5$ and $U_L13$ genes and modulates expression of transduced $U_S1.5$ open reading frame in a cell type-specific manner," *Journal of Virology*, vol. 76, no. 2, pp. 743–754, 2002.

[219] M. Inman, G.-C. Perng, G. Henderson, et al., "Region of herpes simplex virus type 1 latency-associated transcript sufficient for wild-type spontaneous reactivation promotes cell survival in tissue culture," *Journal of Virology*, vol. 75, no. 8, pp. 3636–3646, 2001.

[220] G.-C. Perng, C. Jones, J. Ciacci-Zanella, et al., "Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript," *Science*, vol. 287, no. 5457, pp. 1500–1503, 2000.

[221] M. Ahmed, M. Lock, C. G. Miller, and N. W. Fraser, "Regions of the herpes simplex virus type 1 latency-associated transcript that protect cells from apoptosis in vitro and protect neuronal cells in vivo," *Journal of Virology*, vol. 76, no. 2, pp. 717–729, 2002.

[222] L. Jin, W. Peng, G.-C. Perng, et al., "Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype," *Journal of Virology*, vol. 77, no. 11, pp. 6556–6561, 2003.

[223] G. Henderson, W. Peng, L. Jin, et al., "Regulation of caspase 8- and caspase 9-induced apoptosis by the herpes simplex virus type 1 lantency-associated transcript," *Journal of NeuroVirology*, vol. 8, supplement 2, pp. 103–111, 2002.

[224] I. Schmitz, S. Kirchhoff, and P. H. Krammer, "Regulation of death receptor-mediated apoptosis pathways," *International Journal of Biochemistry and Cell Biology*, vol. 32, no. 11-12, pp. 1123–1136, 2000.

[225] X. Wang, "The expanding role of mitochondria in apoptosis," *Genes and Development*, vol. 15, no. 22, pp. 2922–2933, 2001.

[226] A. Krueger, S. Baumann, P. H. Krammer, and S. Kirchhoff, "FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis," *Molecular and Cellular Biology*, vol. 21, no. 24, pp. 8247–8254, 2001.

[227] D. Carpenter, C. Hsiang, L. Jin, et al., "Stable cell lines expressing high levels of the herpes simplex virus type 1 LAT are refractory to caspase 3 activation and DNA laddering following cold shock induced apoptosis," *Virology*, vol. 369, no. 1, pp. 12–18, 2007.

[228] K. R. Mott, N. Osorio, L. Jin, et al., "The bovine herpesvirus-1 LR ORF2 is critical for this gene's ability to restore the high wild-type reactivation phenotype to a herpes simplex virus-1 LAT null mutant," *Journal of General Virology*, vol. 84, no. 11, pp. 2975–2985, 2003.

[229] G.-C. Perng, B. Maguen, L. Jin, et al., "A gene capable of blocking apoptosis can substitute for the herpes simplex virus type 1 latency-associated transcript gene and restore wild-type reactivation levels," *Journal of Virology*, vol. 76, no. 3, pp. 1224–1235, 2002.

[230] L. Jin, G.-C. Perng, K. R. Mott, et al., "A herpes simplex virus type 1 mutant expressing a baculovirus inhibitor of apoptosis gene in place of latency-associated transcript has a wild-type reactivation phenotype in the mouse," *Journal of Virology*, vol. 79, no. 19, pp. 12286–12295, 2005.

[231] L. Jin, D. Carpenter, M. Moerdyk-Schauwecker, et al., "Cellular FLIP can substitute for the herpes simplex virus type 1 latency-associated transcript gene to support a wild-type virus reactivation phenotype in mice," *Journal of NeuroVirology*, vol. 14, no. 5, pp. 389–400, 2008.

[232] D. Carpenter, G. Henderson, C. Hsiang, et al., "Introducing point mutations into the ATGs of the putative open reading frames of the HSV-1 gene encoding the latency associated transcript (LAT) reduces its anti-apoptosis activity," *Microbial Pathogenesis*, vol. 44, no. 2, pp. 98–102, 2008.

[233] L. A. Samaniego, L. Neiderhiser, and N. A. DeLuca, "Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins," *Journal of Virology*, vol. 72, no. 4, pp. 3307–3320, 1998.

[234] L. A. Samaniego, N. Wu, and N. A. DeLuca, "The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27," *Journal of Virology*, vol. 71, no. 6, pp. 4614–4625, 1997.

[235] J. R. Schlehofer and H. zur Hausen, "Induction of mutations within the host cell genome by partially inactivated herpes simplex virus type 1," *Virology*, vol. 122, no. 2, pp. 471–475, 1982.

[236] L. Yang, C. C. Voytek, and T. P. Margolis, "Immunohistochemical analysis of primary sensory neurons latently infected with herpes simplex virus type 1," *Journal of Virology*, vol. 74, no. 1, pp. 209–217, 2000.

[237] F. J. Branco and N. W. Fraser, "Herpes simplex virus type 1 latency-associated transcript expression protects trigeminal ganglion neurons from apoptosis," *Journal of Virology*, vol. 79, no. 14, pp. 9019–9025, 2005.

[238] M. Joels and E. R. de Kloet, "Control of neuronal excitability by corticosteroid hormones," *Trends in Neurosciences*, vol. 15, no. 1, pp. 25–30, 1992.

[239] B. S. McEwen, "Non-genomic and genomic effects of steroids on neural activity," *Trends in Pharmacological Sciences*, vol. 12, no. 4, pp. 141–147, 1991.

[240] W. P. Halford, B. M. Gebhardt, and D. J. J. Carr, "Mechanisms of herpes simplex virus type 1 reactivation," *Journal of Virology*, vol. 70, no. 8, pp. 5051–5060, 1996.

[241] J. W. Collett and R. E. Steele, "Alternative splicing of a neural-specific *Src* mRNA ($Src^+$) is a rapid and protein synthesis-independent response to neural induction in *Xenopus laevis*," *Developmental Biology*, vol. 158, no. 2, pp. 487–495, 1993.

[242] J. Busser, D. S. Geldmacher, and K. Herrup, "Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain," *Journal of Neuroscience*, vol. 18, no. 8, pp. 2801–2807, 1998.

[243] J. S. Gill and A. J. Windebank, "Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle," *Journal of Clinical Investigation*, vol. 101, no. 12, pp. 2842–2850, 1998.

[244] S. Hu, P. K. Peterson, and C. C. Chao, "Cytokine-mediated neuronal apoptosis," *Neurochemistry International*, vol. 30, no. 4-5, pp. 427–431, 1997.

[245] D. S. Park, S. E. Farinell, and L. A. Greene, "Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons," *The Journal of Biological Chemistry*, vol. 271, no. 14, pp. 8161–8169, 1996.

[246] D. S. Park, B. Levine, G. Ferrari, and L. A. Greene, "Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons," *Journal of Neuroscience*, vol. 17, no. 23, pp. 8975–8983, 1997.

[247] D. S. Park, E. J. Morris, L. A. Greene, and H. M. Geller, "G1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis," *Journal of Neuroscience*, vol. 17, no. 4, pp. 1256–1270, 1997.

[248] D. S. Park, E. J. Morris, L. Stefanis, et al., "Multiple pathways of neuronal death induced by DNA-damaging agents, NGF deprivation, and oxidative stress," *Journal of Neuroscience*, vol. 18, no. 3, pp. 830–840, 1998.

[249] A. A. Nash, A. Jayasuriya, J. Phelan, S. P. Cobbold, H. Waldmann, and T. Prospero, "Different roles for $L3T4^+$ and $Lyt\ 2^+$ T cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system," *Journal of General Virology*, vol. 68, no. 3, pp. 825–833, 1987.

[250] A. Simmons, D. Tscharke, and P. Speck, "The role of immune mechanisms in control of herpes simplex virus infection of the peripheral nervous system," *Current Topics in Microbiology and Immunology*, vol. 179, pp. 31–56, 1992.

[251] T. Liu, Q. Tang, and R. L. Hendricks, "Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection," *Journal of Virology*, vol. 70, no. 1, pp. 264–271, 1996.

[252] C. Shimeld, J. L. Whiteland, N. A. Williams, D. L. Easty, and T. J. Hill, "Cytokine production in the nervous system of mice during acute and latent infection with herpes simplex virus type 1," *Journal of General Virology*, vol. 78, no. 12, pp. 3317–3325, 1997.

[253] W. P. Halford, B. M. Gebhardt, and D. J. J. Carr, "Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1," *Journal of Immunology*, vol. 157, no. 8, pp. 3542–3549, 1996.

[254] A. Simmons and D. C. Tscharke, "Anti-CD8 impairs clearance of herpes simplex virus from the nervous systemml: implications for the fate of virally infected neurons," *Journal of Experimental Medicine*, vol. 175, no. 5, pp. 1337–1344, 1992.

[255] K. M. Khanna, R. H. Bonneau, P. R. Kinchington, and R. L. Hendricks, "Herpes simplex virus-specific memory $CD8^+$ T cells are selectively activated and retained in latently infected sensory ganglia," *Immunity*, vol. 18, no. 5, pp. 593–603, 2003.

[256] T. Liu, K. M. Khanna, B. N. Carriere, and R. L. Hendricks, "Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons," *Journal of Virology*, vol. 75, no. 22, pp. 11178–11184, 2001.

[257] T. Liu, K. M. Khanna, X. Chen, D. J. Fink, and R. L. Hendricks, "$CD8^+$ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons," *Journal of Experimental Medicine*, vol. 191, no. 9, pp. 1459–1466, 2000.

[258] K. Prabhakaran, B. S. Sheridan, P. R. Kinchington, et al., "Sensory neurons regulate the effector functions of $CD8^+$ T cells in controlling HSV-1 latency ex vivo," *Immunity*, vol. 23, no. 5, pp. 515–525, 2005.

[259] L. T. Feldman, A. R. Ellison, C. C. Voytek, L. Yang, P. Krause, and T. P. Margolis, "Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 2, pp. 978–983, 2002.

[260] A. L. Cunningham and Z. Mikloska, "The Holy Grail: immune control of human herpes simplex virus infection and disease," *Herpes*, vol. 8, supplement 1, pp. 6A–10A, 2001.

[261] Z. Mikloska and A. L. Cunningham, "Alpha and gamma interferons inhibit herpes simplex virus type 1 infection and spread in epidermal cells after axonal transmission," *Journal of Virology*, vol. 75, no. 23, pp. 11821–11826, 2001.

[262] D. Kagi and H. Hengartner, "Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses," *Current Opinion in Immunology*, vol. 8, no. 4, pp. 472–477, 1996.

[263] S. Shresta, C. T. Pham, D. A. Thomas, T. A. Graubert, and T. J. Ley, "How do cytotoxic lymphocytes kill their targets?" *Current Opinion in Immunology*, vol. 10, no. 5, pp. 581–587, 1998.

[264] J. E. Knickelbein, K. M. Khanna, M. B. Yee, C. J. Baty, P. R. Kinchington, and R. L. Hendricks, "Noncytotoxic lytic granule-mediated $CD8^+$ T cell inhibition of HSV-1 reactivation from neuronal latency," *Science*, vol. 322, no. 5899, pp. 268–271, 2008.

[265] X. Yang, H. R. Stennicke, B. Wang, et al., "Granzyme B mimics apical caspases: description of a unified pathway for transactivation of executioner caspase-3 and -7," *The Journal of Biological Chemistry*, vol. 273, no. 51, pp. 34278–34283, 1998.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 109239, 28 pages
doi:10.1155/2010/109239

*Review Article*

# A Mechanism of Virus-Induced Demyelination

**Jayasri Das Sarma**

*Department of Biological Sciences, Indian Institute of Science Education and Research-Kolkata, Mohanpur Campus, PO: BCKV Campus Main Office, Mohanpur, Nadia, West Bengal 741252, India*

Correspondence should be addressed to Jayasri Das Sarma, dassarmaj@iiserkol.ac.in

Received 8 January 2010; Accepted 20 March 2010

Academic Editor: Marylou V. Solbrig



Myelin forms an insulating sheath surrounding axons in the central and peripheral nervous systems and is essential for rapid propagation of neuronal action potentials. Demyelination is an acquired disorder in which normally formed myelin degenerates, exposing axons to the extracellular environment. The result is dysfunction of normal neuron-to-neuron communication and in many cases, varying degrees of axonal degeneration. Numerous central nervous system demyelinating disorders exist, including multiple sclerosis. Although demyelination is the major manifestation of most of the demyelinating diseases, recent studies have clearly documented concomitant axonal loss to varying degrees resulting in long-term disability. Axonal injury may occur secondary to myelin damage (outside-in model) or myelin damage may occur secondary to axonal injury (inside-out model). Viral induced demyelination models, has provided unique imminent into the cellular mechanisms of myelin destruction. They illustrate mechanisms of viral persistence, including latent infections, virus reactivation and viral-induced tissue damage. These studies have also provided excellent paradigms to study the interactions between the immune system and the central nervous system (CNS). In this review we will discuss potential cellular and molecular mechanism of central nervous system axonal loss and demyelination in a viral induced mouse model of multiple sclerosis.

## 1. Introduction

Demyelination is the process by which axons lose their normal insulating myelin. Several central nervous system demyelinating disorders have been described in humans including multiple sclerosis, neuromyelitis optica (Devic's disease), acute disseminated encephalomyelitis, and osmotic demyelination (central pontine myelinolysis, extrapontine myelinolysis).

Multiple sclerosis (MS) is a chronic, progressive, or relapsing and remitting demyelinating disorder that affects the central nervous system (CNS) specifically and ranks as a major cause of nervous system disability in young adults aged 20 to 45 [1–3]. It has long been hypothesized that oligodendrocytes (OLGs) and/or the myelin sheath are the target of immune system-mediated destruction in MS. Recent studies have demonstrated that axonal damage [4, 5] also occurs and is likely to be a major component of long-term disability observed in MS. The etiology of MS is not very clearly known but the process of demyelination is believed to involve a Tcell-mediated phenomenon that may be triggered by one or more viral infections. Clinical studies show that infectious agents encountered during adolescence prime the diseases, which appear clinically later in the adult after a variable period of quiescence [6, 7]. Despite numerous attempts, a particular responsible virus has not been identified. Our research has focused on understanding the mechanisms of demyelination. We use the mouse hepatitis virus (MHV) model of murine demyelination in order to dissect the inflammatory and molecular mechanisms of viral-induced demyelination.

## 2. Infectious Etiology of Multiple Sclerosis

The most important evidence that MS might be infectious is based on the fact that the brain and cerebrospinal fluid (CSF) of more than 90% MS patients contain increased amounts of IgG, manifest as oligoclonal bands (OGBs) [8]. There are other but not many CNS disorders in which increased amounts of IgG and OGBs are found. All those diseases are inflammatory, and most are infectious. Furthermore, when the specificity of the increased IgG and OGBs in

those diseases was studied, the IgG in every condition was shown to be antibody directed against the agent that caused disease. For example, the oligoclonal IgG found in subacute sclerosing panencephalitis (SSPE) brain and CSF is directed against measles virus [9] not herpes simplex virus or mumps virus; in cryptococcal meningitis, the IgG is directed against cryptococcus and not another fungus such as candida [10]. These findings provide a rationale for the notion that the oligoclonal IgG in MS brain and CSF is antibody directed against the agent that causes disease.

Moreover, a number of studies have documented viruses as triggers for MS. The fact that viruses are associated with multifocal leukoencephalopathy (PML), SSPE, and postinfectious encephalitis explains the continued interest in viruses as triggers for MS [11, 12]. Studies in herpes virus have been quite extensive, especially in human herpes virus 6 (HHV-6) [13–15] and Epstein-Barr virus (EBV) [16, 17]. Herpes viruses are of particular interest because of their neurotropic, ubiquitous nature and their tendency to produce latent, recurrent infections.

However, there are no studies to date that clearly demonstrate the underlying pathophysiology, correlating the trigger of viral infection with induction of the demyelination process. One key to a better insight into the molecular and cellular mechanisms of MS lies in the development of a reliable animal model. Towards this goal several experimental animal models have been developed to study the mechanisms of virus-induced demyelination.

## 3. Animal Model for Viral- Induced Demyelinating Diseases

Mouse model studies using Theiler's murine encephalomyelitis virus (TMEV) [18, 19] infection and neurotropic strains of Mouse hepatitis virus (MHV) infection have given useful information on putative MS mechanisms [20–23]. In virus-induced demyelination, infection is a necessary prerequisite for demyelination, and the cause/effect relationship makes this model an attractive platform for exploring the etiology and pathogenesis of demyelinating diseases. Chronic viral-induced dem-yelination is associated with viral persistence [24, 25] and concomitant enhancement of major histocompatibility complex class I antigens [26–30]. These features parallel many of the pathologic findings seen in MS, in contrast to monophasic viral or postviral human demyelinating diseases such as acute disseminated encephalomyelitis (ADEM) [31] and PML [32].

## 4. TMEV- Induced Demyelination Versus MHV-Induced Demyelination

Infection of susceptible strains of mice with some strains of TMEV or MHV causes biphasic disease of the CNS, consisting of early acute disease and late chronic demyelinating disease that appears 30 to 40 days post infection (p.i) [33]. As early as 30 days p.i, infected mice develop late chronic demyelinating disease with extensive demyelinating lesions of the white matter and cell infiltrates in the spinal cord, consisting primarily of CD4+ T cells and CD8+ T cells, some monocots/macrophages, and a few B cells and plasma cells. In general, viral infection causes damage to the nervous system by two mechanisms: direct infection of neural cells and immune-mediated tissue injury (immunopathology).

Virtually all types of immune response have been proposed to play important roles in the pathogenesis of TMEV-induced viral clearance and demyelination. It has been proposed that demyelination is caused by an immune-mediated mechanism, in which CD4+ Th1 cells mediate a delayed type of hypersensitivity response with epitope spreading [34]. TMEV-induced CD8+ T cells have been suggested to function as autoreactive cytotoxic cells or regulatory cells [35]. Antibody against TMEV cross-reacts with galactocerebroside, and passive transfer of anti-TMEV antibody can augment demyelination in experimental allergic encephalomyelitis (EAE) [36]. Intracerebral inoculation with a TMEV-infected macrophage cell line induces acute focal demyelination [37], and depletion of macrophages ameliorates TMEV-induced demyelination [38].

Evidence from highly neurovirulent *JHM* strains of MHV suggests that MHV-induced demyelination is also primarily immune mediated [39, 40]. Demyelination can be completely eliminated in JHM-infected, RAG $-/-$ mice that lack functional T and B cells, and this process can be reversed upon transfer of splenocytes from immunocompetent mice [41]. It has also been shown by depletion and transfer studies in the JHM model that either CD4+ or CD8+ T cells can induce demyelination.

However, MHV-A59-induced demyelination has been shown to develop in the absence of B and T cells [42]. Furthermore, depletion of CD4+ or CD8+ T cells after the acute stage of infection does not reduce demyelination [43]. Thus, different related strains of MHV may induce demyelination via unique mechanisms, and it is likely that in the absence of an intact immune response, some strains of MHV infection in the CNS are responsible for the onset of demyelination, possibly through the direct destruction of OLGs. MHV-induced demyelination can serve as a model for oligodendroglial tropic, nonimmune-mediated mechanisms of demyelination that may have important relevance for our understanding of MS.

## 5. MHV Pathobiology

MHV is a member of the coronavirus family [44–46]. The virus infects many vertebrate hosts and induces a variety of diseases ranging in severity. The outcome and degree of MHV-induced disease are dependent on several factors, including the age and strain of the mouse, the strain of MHV, and the route of virus inoculation. Even very closely related strains of MHV differ in pathogenic properties. Some strains of MHV are purely hepatotropic (e.g., MHV-2) [47]; some are primarily neurotropic (e.g., JHM, MHV-4, an isolate of JHM) [22, 48]; while others (e.g., MHV-A59 and MHV3) [21, 49] are both hepatotropic and neurotropic.

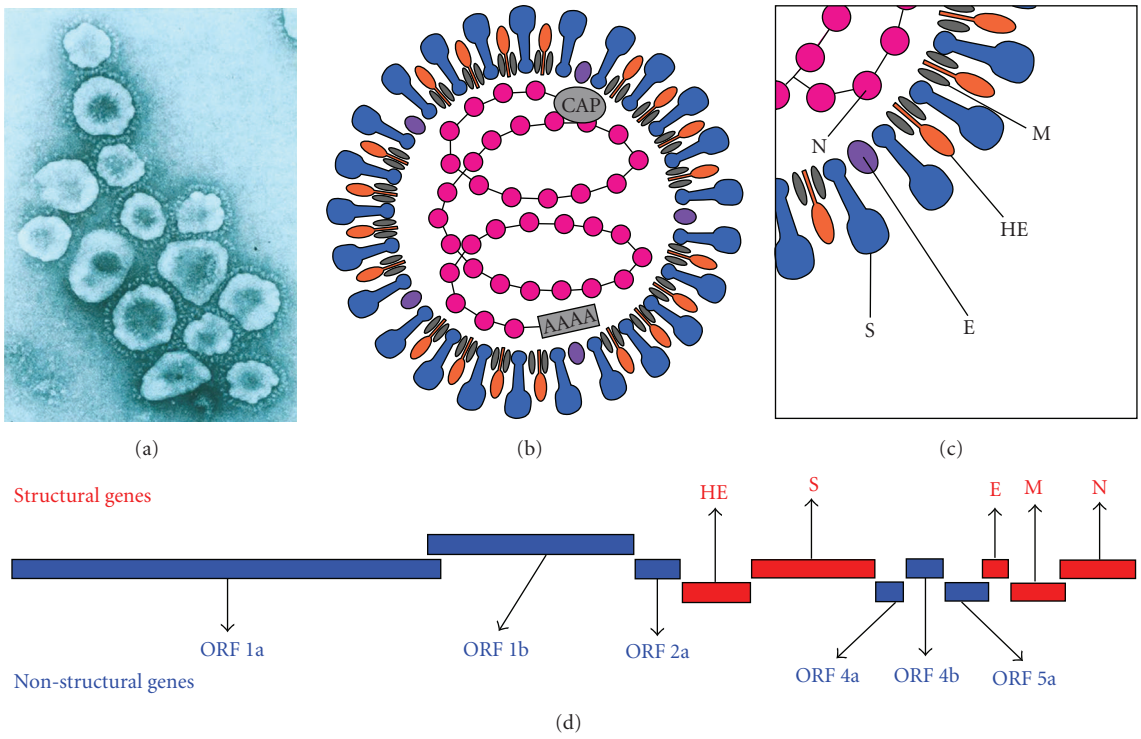



Figure 1: MHV virion and MHV genome: (a) electron micrographs of coronavirus particle. (b, c) Schematics of structural protein organization in the MHV virion; MHV virion is somewhat pleomorphic, containing an internal helical RNA genome-nucleocapsid phosphoprotein (N) complex, surrounded by an envelope containing glycoprotein peplomer (S, E, HE, and M) as specified in the text. (d) Organization of the genes in the MHV genome; structural genes are shown in red letters and nonstructural genes in blue letters.

## 6. MHV Virions

MHV virions are 60–200 nm in diameter, somewhat pleomorphic (Figure 1(a)), containing an internal helical RNA genome-nucleocapsid phosphoprotein (N) complex, surrounded by an envelope containing Spike glycoprotein (S), transmembrane glycoprotein (M), envelope protein (E), and hemagglutinin-esterase protein (HE) (Figures 1(b) and 1(c)) [50]. Structural proteins expressed at the 3′ half of the MHV genome are believed to be responsible for MHV pathogenesis. S is a highly glycosylated protein. The outward radial projection of S gives the virus its family name *corona* (like a crown). HE forms smaller spike on virions, but its function in the virus life cycle is yet not clearly defined. Both M and E play a role in the assembly of virions [51]. The virions also have the I protein of unknown function [52].

## 7. MHV Genome

The MHV genome is a singlestranded, and non-segmented, polyadenylated RNA, of positive polarity [53]. It is one of the largest known viral RNA genomes with a length of 31-32 Kb [46, 54, 55]. The RNA genome contains 7 genes, termed 1 to 7, encoding structural as well as nonstructural genes (Figure 1(d)). There is a leader RNA sequence at the 5′ end of the genome that regulates the transcription of MHV-RNAs. The next two-thirds of the 5′ end of the genome are covered by Gene 1, which encodes a set of polyproteins with polymerase and replicase functions. Gene 2 encodes two proteins including a 30 kD product of ORF2a. Gene 2 also transcribes a 65 kD hemagglutinin-esterase protein in some MHV strains. Genes 3, 6, and 7 code for the spike protein (S), transmembrane glycoprotein (M), and nucleocapsid protein (N), respectively. Gene 4 encodes a 12–14 kD product while gene 5 encodes a 13 kD product of a still unidentified nonstructural protein and a membrane-associated E protein. It also contains a large open reading frame embedded entirely in the 5′ half of its N gene. This internal gene (I) encodes a mostly hydrophobic 23 kD structural protein [52].

## 8. MHV Entry and Replication

During lytic infection, MHV enters the host cell via attachment of the S protein to specific receptors on the host pericellular surface. Carcinoembryonic antigen is one receptor implicated in infection of cultured mouse cells [56]. The receptor interaction triggers fusion of the viral and plasma membranes, allowing entry of the nucleocapsid into the cytoplasm, where all virus-specific RNA and proteins are synthesized. The nascent MHV virions acquire their membrane envelope by implanting themselves into the lumen of the intermediate compartment between the endoplasmic reticulum and Golgi complex of the host. Mature virions are thought to move through the vesicles via the secretory pathways and exit the cell when the vesicles fuse with the plasma membrane [57].

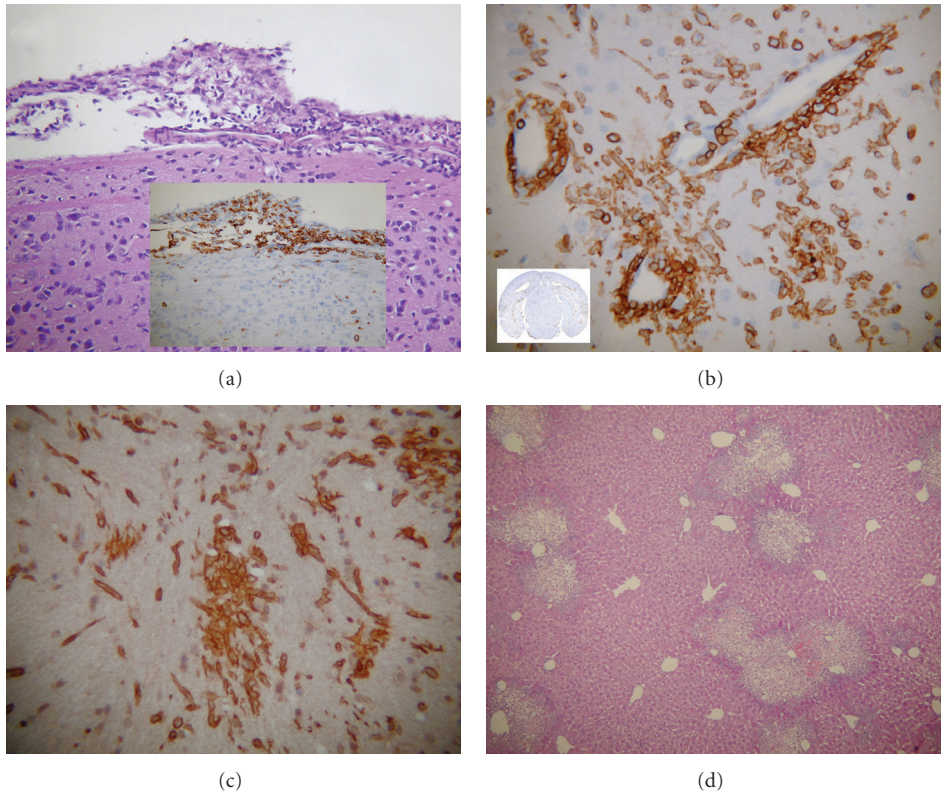


Figure 2: Archetypical histopathology of brain and liver during acute infection with neurotropic strain. MHV-A59: serial coronal sections from MHV-A59-infected mice at day 5 post infection were stained with H & E or immunostained for CD45 (LCA: leukocyte common antigen, marker for inflammatory cells) or microglia/macrophage marker CD11b (OX42). (a) H & E staining shows acute meningitis and inset shows the presence of inflammatory cells positive for LCA. (b) Coronal sections stained for LCA show acute encephalitis in brain section characterized by the formation of perivascular cuffing in high magnification. Inset shows presence of inflammatory infiltrates throughout the parenchyma. (c) The majority of LCA-positive inflammatory cells are stained positive for the macrophage/microglia marker CD11b in MHV-A59-infected brain parenchyma. (d) Liver pathology from the same infected mouse stained with H & E shows lesions throughout the liver section.

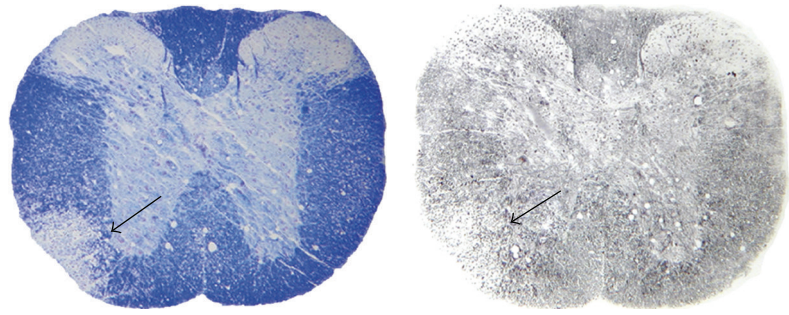
## 9. MHV Recombination

Viral recombination has been extremely useful for systematically exploring the possible biological consequences of viral genomic differences. The recombination technique has been extensively exploited to study reovirus disease in mice, where the segmented nature of the viral RNA has enabled resorting of viruses with RNA segments from different strains with different biological properties [58]. In sindbis virus and picornaviruses, a full-length infectious clone of the viral genome has been exceptionally useful in the study of viral properties [59, 60].

In MHV, the replicase carries out "discontinuous transcription" in the fusion of body and leader sequences in subgenomic RNAs and also during recombination events which occur at high frequency during MHV replication. High-frequency RNA-RNA genomic recombination events are archetypal for coronaviruses [61]. These serve as mechanisms of virus evolution and modulation of viral pathogenesis, a largely unexplored area of study which offers deep insight into the genomic control of biological properties. But for a long time the large genome size was a technical obstacle to achieving such a goal with coronaviruses. This problem was circumvented by molecularly cloning defective interfering-like RNA, leveraging the high RNA recombination frequencies during mixed infection. This targeted recombination technique in MHV was developed by Dr. Paul S. Masters and colleagues and used extensively to introduce alterations into the 3′ end of the MHV genome [62–64]. It was able not only to exchange specifically the structural and non structural genes among the MHV strains but also to introduce single amino acid substitutions. In the last few years there have been reports of full-length infectious clones for many coronaviruses including MHV and a human coronavirus causing severe acute respiratory syndrome (SARS) [65–69].

## 10. MHV Pathogenesis

*10.1. Meningoencephalitis, Demyelination, and Axonal Loss.* Upon intracranial (i.c) infection of neurotropic MHVs acute meningoencephalitis (with or without hepatitis) is the major pathologic process (Figure 2). Viral titer reaches its peak at days 3 and 5 post infection (p.i) [47]. Infectious virus is cleared within the first 10–14 days; however, at this time mice begin to develop demyelination, either clinical or



(a) (b)

Figure 3: Demyelination and axonal loss in a neurotropic strain of MHV, *MHV-A59-infected mouse spinal cord.* Serial cross-sections (5 $\mu$m thick) from MHV-A59-infected mouse spinal cord at day 7 p.i. were stained for myelin with LFB or by Bielchowsky silver impregnation. (a) A large demyelinating plaque observed in MHV-A59-infected mouse spinal cord is shown (arrow indicates demyelinated area). (b) In an adjacent section, the same demyelinating plaque of MHV-A59-infected spinal cord shows loss of axons (arrow indicates area of axonal loss) (adapted from the work of [73]).

accompanied by chronic hind limb paralysis [21, 70]. Both MHV-JHM and MHV-A59 cause inflammatory demyelination in the brain and spinal cord (Figure 3(a)) whereas MHV3 only causes vasculitis [49, 71].

It was formerly believed that in primary MHV-induced demyelination neuronal axons remain relatively preserved. Recently, it has been observed that adoptive transfer of spleen cells from immune MHV B6 mice into MHV-infected RAG − / − mice (that are defective in recombinase activating gene 1 expression, and thus lack mature B or T cells) resulted in demyelination with increased axonal damage [72]. They also showed that axonal damage is, in large part, immune mediated in MHV-infected mice and occurs concomitantly with demyelination. Concurrent axonal loss and demyelination have recently also been observed with S protein recombinant DM strain-infected mouse spinal cord as shown in Figure 3 ((a) demyelination; (b) axonal Loss) [73].

*10.2. Role of Immune Cells in Viral Clearance and Demyelination.* MHV clearance requires both CD8+ and CD4+ T cells [74–76]. CD4+ T cells are necessary for proper CD8+ T cell activation, survival, and retention in the infected CNS [75]. Clearance of infectious virus is mediated by both cytolytic- and cytokine-mediated mechanisms. Exact mechanisms of MHV-induced demyelination are unclear although both macrophages and T cells modulate pathologic changes [41, 77]. Severe combined immunodeficient (SCID) mice, Rag-1 knockout mice, and UV-irradiated mice infected with MHV-JHM have few lesions 7–15 days p.i despite viral replication [40, 41, 76–78]. These data suggest that lymphocytes are required for demyelination. However, demyelination mediated by $\gamma\delta$T cells still occurs in nude mice that lack CD4+ and CD8+ T cells [79]. Interestingly, demyelination occurs to a similar extent in wild type, B cell-deficient, and Rag-1 knockout mice and in mice lacking antibody receptors or active complement pathways, when infected with MHV-A59 [42].

*10.3. Viral RNA Persistence During Chronic Demyelination.* Viral RNA persistence is essentially the failure of the immune system to clear viral RNA from infected organs, mainly the CNS. Viral RNA persistence has been demonstrated in infections with all of the neurotropic demyelinating strains of MHV including JHM and MHV-A59 [24, 25, 80]. Viral RNA persistence appears to be an important factor and perhaps even a prerequisite for MHV-induced demyelination during chronic immune-mediated demyelination. MHV strains that do not persist, such as MHV-2 and Penn 97-1 (laboratory recombinant strains of MHV) [81, 82], also do not demyelinate. However, the persistence-positive, demyelination-negative phenotypes of Penn 98-1 and Penn 98-2 (S protein recombinant strain of MHV) [81] indicate that viral persistence per se is insufficient to induce demyelination. Penn 98-1 and Penn 98-2 (S protein recombinant strain) [81] may persist in neuronal cells of gray matter while demyelinating strains, such as MHV A59, persist in white matter, suggesting that cell-specific persistence is necessary for demyelination. During chronic infection, MHV RNA persistence in the white matter has previously been demonstrated [25]. However, it is not known which glial cells are mainly responsible for the induction of demyelination.

*10.4. Glial Cell Interaction in the Induction of Demyelination.* A temperature-sensitive demyelinating mutant of JHM is known to infect mainly nonneuronal cells and specifically to have a strong affinity for astrocytes as well as to cause white matter lesions in the mouse [83, 84]. On the other hand, neurotropic nondemyelinating MHV3 has an in vitro affinity for neurons, ependymal cells and meningothelial cells but not for astrocytes or oligodendrocytes. MHV3 can induce an initial ependymitis, meningitis, and encephalitis in the absence of white matter lesions [49]. These observations reinforce the importance of glial cell infection in the onset of demyelination. Astrocytes and microglia play an important defensive role in MHV pathogenesis by secreting cytokines

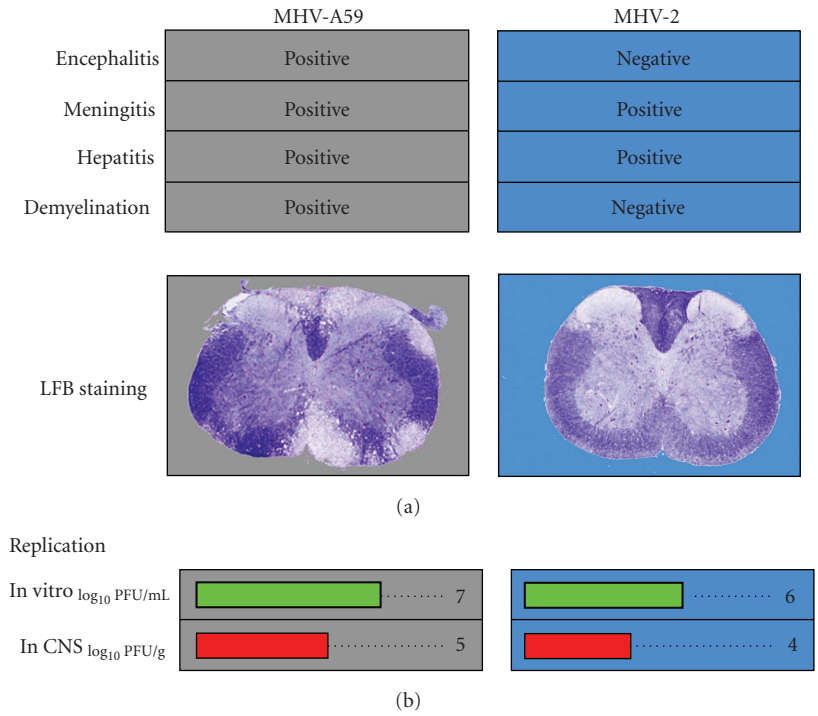



Figure 4: Index of differential pathogenesis and replication of archetypical neurotropic strain, MHV-A59 and nonneurotropic primarily hepatotropic strain, MHV-2.

[85–87]. Cytokines are believed to aid in host defense against MHV by contributing to elimination of the virus from the CNS. It has been reported that specific cytokines and chemokines (such as Interferon $\alpha$, $\beta$, and $\gamma$), tumor necrosis factor (TNF-$\alpha$), IL-2, Il-6, IL-1$\alpha$, and IL-1$\beta$ are all induced during the acute stage of MHV infection in the CNS [88]. TNF-$\alpha$, IL-6, and IL-1$\beta$ are reportedly secreted by astrocytes of persistently infected spinal cords [89]. The role of individual CNS cells in the induction of demyelination remains to be further elaborated.

## 11. Comparative Characterization of Natural and Recombinant Demyelinating (DM) and Nondemyelinating (NDM) Strains of MHV to Understand the Mechanisms of Demyelination and Axonal Loss

Natural and genetically constructed recombinant MHV strains (generated by targeted RNA recombination) with differential pathological properties were used to understand the mechanisms of demyelination and concomitant axonal loss. These encompass both demyelinating (DM) and nondemyelinating (NDM) strains of MHVs, on which we have performed comparative studies correlating the phenotypes, genomic sequences, and their pathogencity. As a first step mice were inoculated mice with plaque-purified demyelinating strain MHV-A59 [21] and nondemyelinating strain MHV-2 [90]. MHV-A59, a wild type parental strain of MHV, infects a variety of cell types, including neurons, astrocytes, oligodendrocytes, microglia, and ependymal cells [21, 70, 91, 92] in the central nervous system (CNS) and causes acute meningitis, encephalitis, hepatitis, and chronic demyelination. In contrast, MHV-2, a closely related fusion negative [93] strain to MHV-A59, has limited ability to invade brain and spinal cord, causing meningitis without encephalitis or demyelination [47, 82] but causing severe hepatitis (Figure 4), making it an appropriate experimental negative control for our understanding of mechanisms of MHV-A59-induced CNS inflammatory disease processes.

*11.1. Inflammation in the Brain.* Anti-CD45 (LCA: leukocyte common antigen) staining confirmed that the increased cells in the meninges of MHV-A59 (Figure 5(a)) or MHV-2-infected CNS (Figure 5(b)) consist predominantly of infiltrating inflammatory cells (Figures 5(c) and 5(d)). MHV-A59 also induced focal acute encephalitis, characterized by inflammatory infiltrates in brain parenchyma with perivascular cuffing (Figures 5(e) and 5(g)) whereas, MHV-2-infected brain inflammatory cells were restricted to the meninges, choroid plexus, and subependymal region with minimal, if any, invasion of brain parenchyma (Figures 5(f) and 5(h)). The majority of infiltrating cells were CD11b+, a macrophage/microglia marker (Figures 5(i) and 5(j)). Some CD3-stained infiltrating T cells were also found (data not shown) although nonspecific background staining of neurons with available anti-CD3 antibodies made staining difficult to quantify. Together, data suggests that MHV-induced CNS inflammation consists of mixed inflammatory cells, predominantly macrophages/microglia [73, 94].

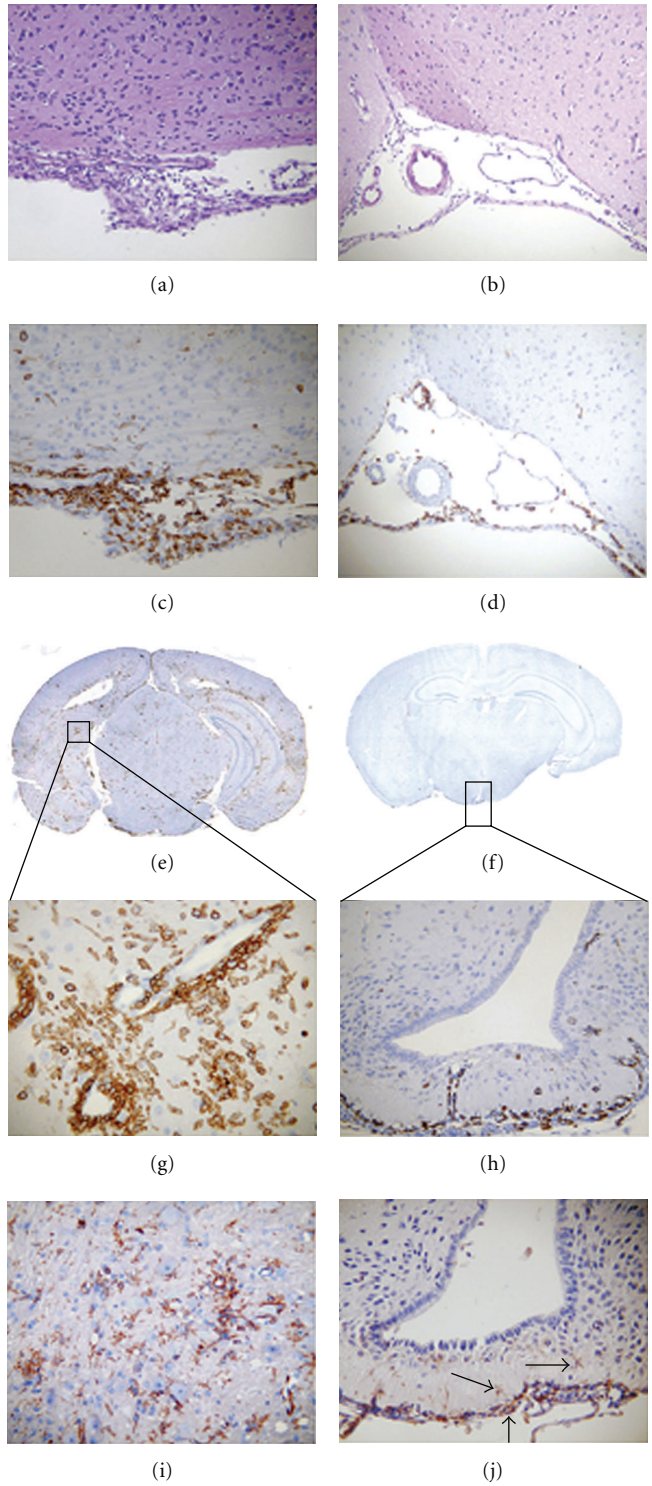



FIGURE 5: Histopathology of brain sections during acute infection with MHV-A59 compared to MHV-2. 5 $\mu$m thick serial coronal sections from MHV-A59- and MHV-2-infected mice at day 5 post inoculation were stained with H & E (a, b) or immunostained for LCA (c–h) or CD11b (i, j). Histopathology shows acute meningitis characterized by brisk leptomeningeal inflammatory infiltration in the subarachnoid space of the basal forebrain in both MHV-A59- (a, c) and MHV-2-infected (b, d) mice. Coronal sections stained for LCA show acute encephalitis in MHV-A59-infected mouse brain characterized by the presence of inflammatory infiltrates throughout the parenchyma (e) and formation of perivascular cuffing (g) in high magnification whereas in MHV-2 infection infiltrating inflammatory cells were rarely observed in brain parenchyma (f) and, when present, were restricted to the subependyma (h). The majority of LCA+ inflammatory cells stained positive for the macrophage/microglia marker CD11b in MHV-A59-infected brain parenchyma (i) and the few positive cells in MHV-2-infected brain (j) remained restricted to subependyma (arrows). Original magnifications for (a–d) and (h) are 100x, for (g, i, and j) are 200x, and for (e) and (f) are laser-scanned images. (adapted from the work of [94]).

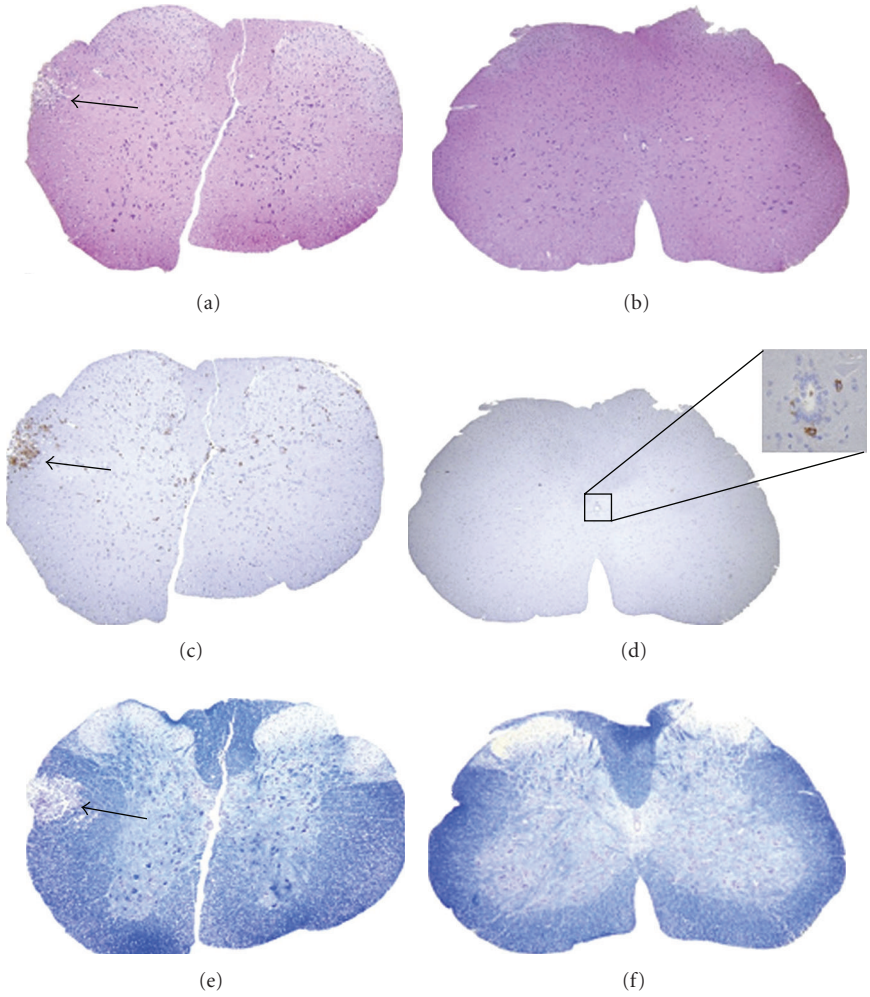



Figure 6: Comparative histopathology of MHV-A59- and MHV-2-infected mouse spinal cords. Serial cross-sections (5 $\mu$m thick) from MHV-A59- and MHV-2-infected mouse spinal cord at day 5 post inoculation were stained with H & E (a, b), LCA (c, d), or LFB (e, f). Several inflammatory lesions (a) with infiltrating LCA+ cells (c) were observed in MHV-A59-infected mouse spinal cord (arrows indicate lesion area) whereas in MHV-2-infected mouse spinal cord no parenchymal lesions were observed (b), with only rare LCA+ infiltrates observed near the central canal (d). An adjacent section of MHV-A59-infected spinal cord shows loss of myelin by LFB staining ((e) arrow indicates demyelinated area) whereas normal myelin was observed in MHV-2-infected mouse spinal cord (f). Original magnification of (a–f) is 40x. (adapted from the work of [94]).

*11.2. Inflammation in the Spinal Cord.* Pathology was also assessed in cross-sections of spinal cord from cervical, thoracic, and lumbar regions. Similar to brain, H & E and LCA staining demonstrated that both MHV-A59 and MHV-2 induced meningitis whereas only MHV-A59 induced myelitis (Figures 6(a) and 6(c)). In MHV-2 infection, inflammatory cells were rare and restricted near the ependymal cell lining of the central canal (Figures 6(b) and 6(d)). Similar to the brain, the majority of inflammatory cells in MHVA59 were CD11b+ (Figure 6(c)). Luxol fast blue (LFB) staining was performed to visualize myelin [94]. Demyelinating plaques developed as early as day 5 post inoculation in MHV-A59-infected mice (Figure 6(e)), with no demyelination in MHV-2-infected mice (Figure 6(f)). Day 30-post inoculation tissue sections from MHV-A59-infected mice showed a similar pattern of demyelination as on day 5, but the number and area of plaques were larger, and MHV-2-infected mice did not exhibit any demyelination. To avoid a high mortality rate of MHV-2 due to hepatitis, 0.5 LD50 doses (50 PFUs) were used. However, to ensure that the inability of MHV-2 to cause encephalitis or demyelination is not dose dependent, mice were also inoculated with MHV-A59 at 50 PFUs. MHV-A59 produced larger demyelinating lesions given at 2,000 PFUs than at 50 PFUs, but with both doses, 100% of mice were affected. Results confirm earlier findings that MHV-A59 induces demyelination whereas MHV-2 is nondemyelinating at day 30 [47]. Demyelination was not previously assessed at earlier time points. Demyelination begins as early as day 5 post inoculation, indicating that MHV-A59-induced myelin damage begins at the time of acute inflammation, similar to what is observed in MS and EAE lesions [95].

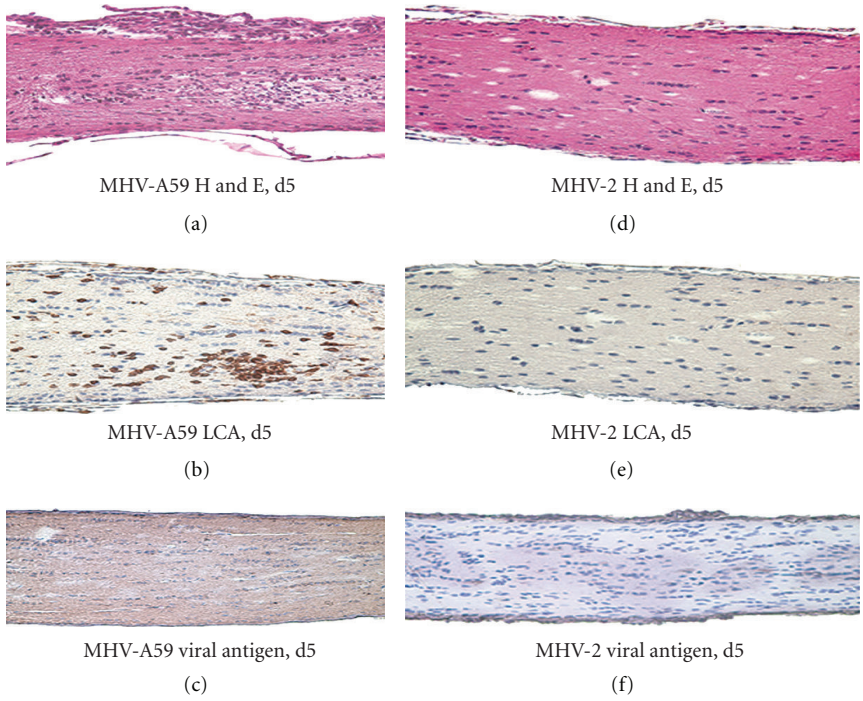



Figure 7: Optic nerve histopathology from MHV-A59-infected and MHV-2-infected mice. Longitudinal sections (5 $\mu$m thick) of optic nerve from MHV-infected mice are shown. Numerous inflammatory cells are evident in an MHV-A59-infected optic nerve with ON 5 days post inoculation stained by H & E (a) and LCA (b). Optic nerve, 5 days post inoculation, with MHV-2 (d) shows a lack of inflammation, with no LCA staining (e) detected. Viral antigen staining of optic nerve from an MHV-A59-infected mouse (c) shows low-level axonal staining whereas no viral antigen is detected in MHV-2-infected (f) mouse optic nerve. Photographs shown at original magnification x20. (adapted from the work of [94]).

*11.3. Optic Nerve Inflammation.* The presence of LCA+ inflammation and associated demyelination in brain and spinal cord led to hypothesize that MHV-A59-infected mice may develop optic neuritis (ON) similar to experimental ON in EAE mice. Optic nerves from MHV-A59- and MHV-2-infected mice were cut into 5 $\mu$m longitudinal sections and stained with H & E and inflammatory cell markers. For comparison, relapsing EAE was induced in 8-week-old SJL/J mice by immunization with proteolipid protein peptide as described previously in [96]. Mice were sacrificed on day 11 post immunization, when incidence of ON peaks and optic nerves were isolated. Inflammatory cells infiltrating the optic nerve sheath and parenchyma are shown by H & E and LCA staining (Figures 7(a) and 7(b)) 5 days post inoculation with MHV-A59, similar to inflammation seen in EAE ON [94]. In contrast, optic nerves from MHV-2-infected mice did not develop ON (Figures 7(d) and 7(e)), with histological appearance similar to control optic nerves. Both CD11b+ and fewer CD3+ cells were noted in MHV-A59-infected optic nerves similar to brain. By day 15 post MHV-A59 infection, optic nerve inflammation completely resolved. Detection of viral antigen in optic nerves was limited to axons because there are no neuronal cell bodies present. Light diffuse staining detected in optic nerves from MHV-A59-infected mice (Figure 7(c)), but not MHV-2-infected mice (Figure 7(f)), suggests that MHV-A59 viral antigen is likely present in optic nerve axons. Consistent with the observed pattern of inflammation, optic nerves from MHV-A59-infected mice had areas of demyelination detected by LFB staining 30 days post inoculation whereas no demyelination occurred in MHV-2-infected mice. The degree of optic nerve inflammation was scored on a 0 (no inflammation) to 4 (severe inflammation) point scale described previously in [96–98], with any amount of inflammation (score 1–4) considered positive for ON. ON was detected as early as 3 days post inoculation with MHV-A59, with peak incidence at day 5 (Figure 8(a)) and resolution by day 15. At the peak of ON (day 5), almost 50% (10 of 21) of optic nerves examined from MHV-A59-infected mice had ON (Figure 8(b)), with an average relative inflammation score of $1.7 \pm 0.21$ whereas, only one of 21 nerves from MHV-2-infected mice had even mild inflammation (score 1.0). Incidence of ON in EAE was 60% (6 of 10) with a $1.83 \pm 0.31$ average inflammation score, similar to prior studies [96, 98] and comparable to the incidence induced by MHV-A59 infection.

*11.4. Axonal Loss and Demyelination in the Optic Nerve.* To examine whether MHV-A59-induced ON also leads to axonal loss, optic nerves were stained by Bielschowsky silver impregnation, and the area of axonal staining was quantified as described previously [99]. 30 days post inoculation, MHV-A59-infected mice had significantly decreased axonal staining compared to control nerves or nerves from MHV-2-infected mice (Figure 8(c)).

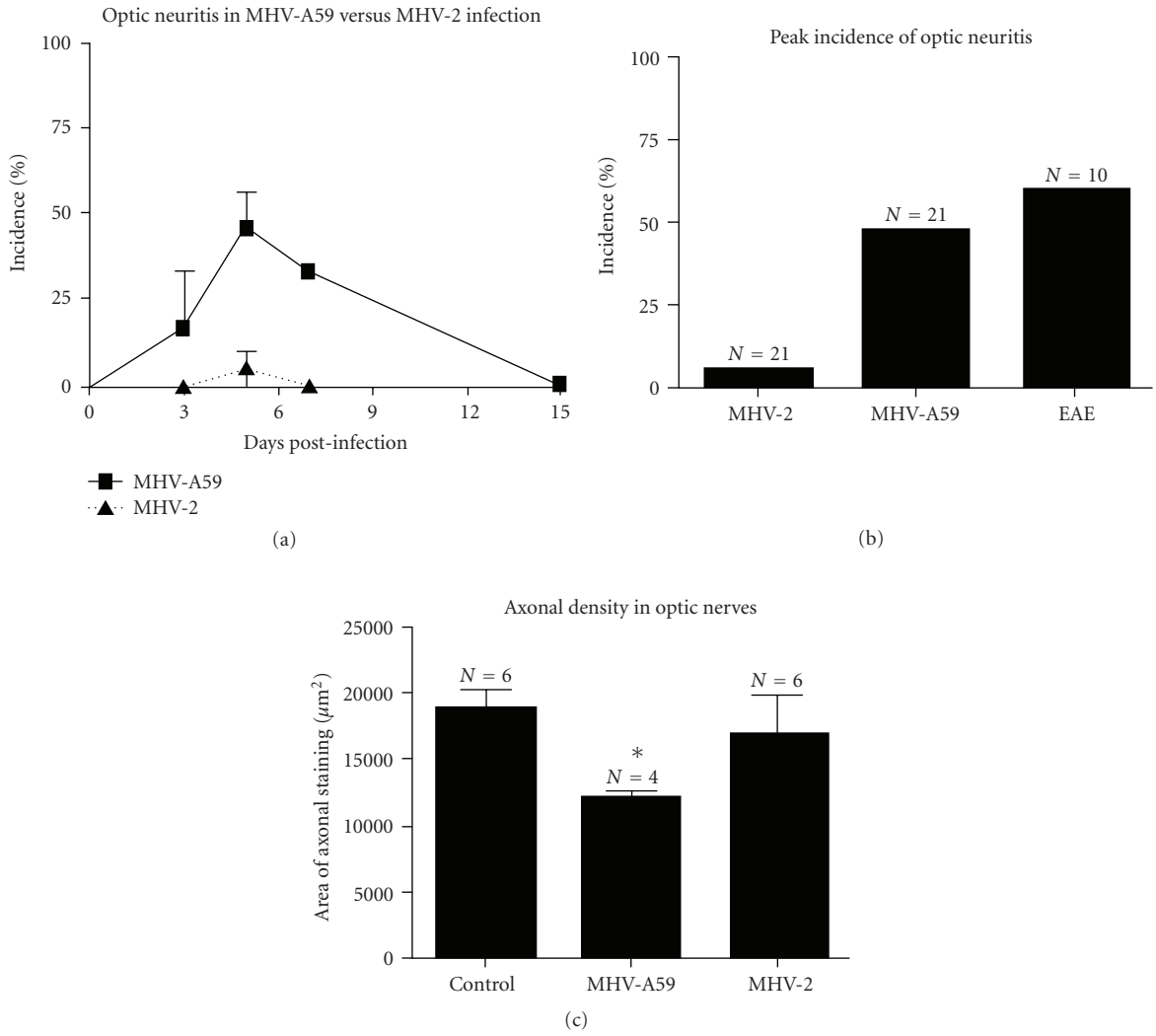



Figure 8: Occurrences of ON and axonal loss in MHV-infected and EAE mice. (a) ON, determined by presence of any level of inflammation in the optic nerve, is detected as early as day 3 post inoculation with MHV-A59. Incidence increases by day 5, persists at day 7, and resolves by day 15. In contrast, almost no MHV-2-infected mice develop ON over the same time course. Mean ± SEM incidence from 3 experiments is shown. (b) The peak incidence of MHV-A59-induced ON, detected at day 5 postinoculation, was 48% (10 of 21 nerves) whereas only one of 21 nerves from MHV-2-infected mice developed ON. 60% (6 of 10) of optic nerves from EAE (experimental autoimmune encephalitis; autoimmune model for demyelination) mice develop ON at the peak of inflammation on day 11 post immunization. (c) The area of axonal staining (mean ± SEM) detected by Bielschowsky silver impregnation is significantly lower in optic nerves from MHV-A59-infected mice as compared to optic nerves from control, uninfected mice or MHV-2-infected mice ($^*P \leq .05$). (adapted from the work of [94]).

## 12. Summary of Comparative Inflammatory Neuropathology of DM (MHV-A59) and Nondemyelinating (MHV-2) Strain of MHV: Insight for Understanding the Mechanisms of Demyelination and Axonal Loss

Comparative studies demonstrate that MHV-A59, but not MHV-2, induces demyelination in the CNS during acute encephalitis, as early as 5 days post inoculation, in addition to chronic demyelination previously observed at day 30 [47]. Inflammation triggered by MHV infection consists of mixed inflammatory cells, predominantly macrophages/microglia, which differs somewhat from immune-mediated models of MS where infiltrating T cells are significant contributors to pathology [95, 100]. Importantly, MHV-A59 also induces ON with similar severity and incidence as seen in the autoimmune antigen-triggered model EAE. Experimental ON is an important model being used to characterize neuronal damage and develop novel therapies for MS [98, 101–103], but studies have shown that different EAE models, induced by distinct antigens, lead to different mechanisms of RGC loss [96, 104]. The MHV-A59-induced ON model will provide a critical adjunct to study the pathophysiology of ON secondary to viral-mediated inflammation as this is one mechanism that can cause ON and MS in human patients.

## 13. Genomic Sequence Comparison between DM (MHV-A59) and NDM (MHV-2) Strain Provides Insight for Putative Genomic Control of Neuropathogenic Properties

Sequence comparison between MHV-2 (gene bank accession number: AF201929) and MHV-A59 (gene bank accession number 9629812) revealed 94%–98% sequence homology of the replicase genes, 83%–95% sequence homology of genes 2a, 3, 5b, 6, and 7, and a marked difference in the sequence of genes 2b, 4, and 5a among two strains. Among the structural proteins the S protein interacts with the host receptor and mediates viral-cell membrane fusion during viral entry [105]. The ~1300 amino acid spike protein in MHV-A59 is usually posttranslationally cleaved into two domains which associate with each other to form a functional dimer. In the S protein from MHV-2 strain, however, this cleavage does not occur, the reasons for which are not clearly evident. A look at the pairwise alignment between the S proteins from MHV-A59 and MHV-2 reveals that around 84% residues are common in both the chains and an additional 10% residue is similar. The 6% residues with no identity or similarity, include a 43-residue insertion in MHV-2. This insertion is by far the most significant difference in the sequence.

The role of S protein as agent of organ tropism and pathogenesis was hypothesized from comparative studies of different naturally occurring MHV strains [91, 106]. Nucleotide sequencing revealed that alterations in virus virulence were most closely associated with differences in the S gene. These findings were reinforced using targeted RNA recombination to exchange specific gene/genes of interest between different strains of MHV [107–112]. Several targeted RNA recombination studies have directly demonstrated that the S gene is a major determinant of virulence of MHV in mouse brain and liver.

## 14. Construction of Isogenic Recombinant Strains of MHV Expressing DM and NDM Strain S Protein

To determine whether the S gene of MHV also contains determinants of demyelination and whether demyelination is linked to encephalitis and chronic stage viral persistence, targeted RNA recombination was used to create new MHV strains, Penn 98-1 and Penn 98-2, by replacing the S gene of the encephalitic and DS (MHV-A59) with the S gene of a closely related, nonencephalitic NDS (MHV-2) [81]. Molecularly cloned vector pMH54 [111, 113], which contains the entire 3' end of the genome from MHV-A59, was used for construction of the recombinant viruses. Comparative pathological studies between recombinant DS (wtR13) and the NDS Penn 98-1 and Penn 98-2 demonstrated that Penn 98-1 and Penn 98-2 exhibit similar inflammatory infiltration (encephalitis) (Figures 9(a) and 9(b)) in the CNS during the acute stage. While wtR13 can induce demyelination at day 30 p.i. (Figure 9(c)), Penn 98-1 and Penn 98-2 are unable to induce demyelination (Figure 9(d)). Penn 98-1 and Penn 98-2 also exhibited chronic stage persistence in the spinal cord [81]. For convenience and clarity, in subsequent sections these recombinant strains were labeled in different names though they are the same but presented in different names, Penn 98-1 and Penn 98-2 are referred to as RSMHV2 (viruses expressing the MHV-2 spike in the MHV-A59 background) and wtR13 as RSA59 (viruses expressing the MHV-A59 spike in the MHV-A59 background).

As alteration of the MHV S protein influences pathogenesis, it is important to understand how alteration of the S protein alters the virus-host interaction. Previously this type of study was carried out using specific antibodies that detect viral antigen in tissues. Targeted recombination was used to select MHV isolates with stable and efficient expression of the gene encoding EGFP to facilitate the in vivo detection of virus in the mouse CNS as well as to trace the viral entry and spread in tissue culture [114]. The EGFP gene was inserted into the MHV genome in place of the nonessential gene 4, as interruption of the ORF 4 did not decrease neurovirulence in JHM [115]. The viruses replicated with similar kinetics as wild-type virus both in tissue culture and in the mouse CNS. They caused similar encephalitis and demyelination in animals as the wild-type virus or their recombinant strains; however, they were somewhat attenuated in virulence. Isogenic EGFP-expressing viruses differ only in the S gene and express either the S gene of the highly neurovirulent. Previously used names for $RSJHM_{EGFP}$ and $RSA59_{EGFP}$ are $S_4R_{EGFP}$ and $S_{A59}R_{EGFP}$, respectively. JHM spike-mediated neurovirulence was associated with extensive viral spread in the brain [114]. The difference in virulence and patterns of spread of viral antigen between the two isogenic recombinant strains reflected the differences between parental viruses expressing each of these S genes [81, 107]. These EGFP-expressing viruses are powerful tools that can be used to follow viral spread over time without terminating infection by the fixation necessary for immunofluorescence. In order to further compare the differential pathogenesis and the CNS cell tropism between DM and NDM strain of MHV, an EGFP-expressing NDS of MHV $RSMHV2_{EGFP}$, which contains the MHV-2 (NDS) S gene in the MHV-A59 background, was constructed [114]. A cartoon representation of construction of EGFP-tagged strains of MHV has been shown in Figure 10. Detailed molecular studies show the EGFP gene is inserted and expressed at high levels that can be readily detected by microscopy [114].

## 15. EGFP Expressing DM and NDM Strain of MHV: A Tool to Study the Mechanisms of MHV-Induced Neuroinflammation

Frozen sagittal brain sections were obtained from $RSA59_{EGFP}$- and $RSMHV2_{EGFP}$-infected mice, postfixed, and observed by fluorescent microscopy. One of such representative EGFP expressing sagittal brain sections has been shown in Figure 11. EGFP fluorescence was observed in similar regions of the brain in mice infected with both the viruses; however, the number of infected cells differed between strains. Despite the similarity in regional

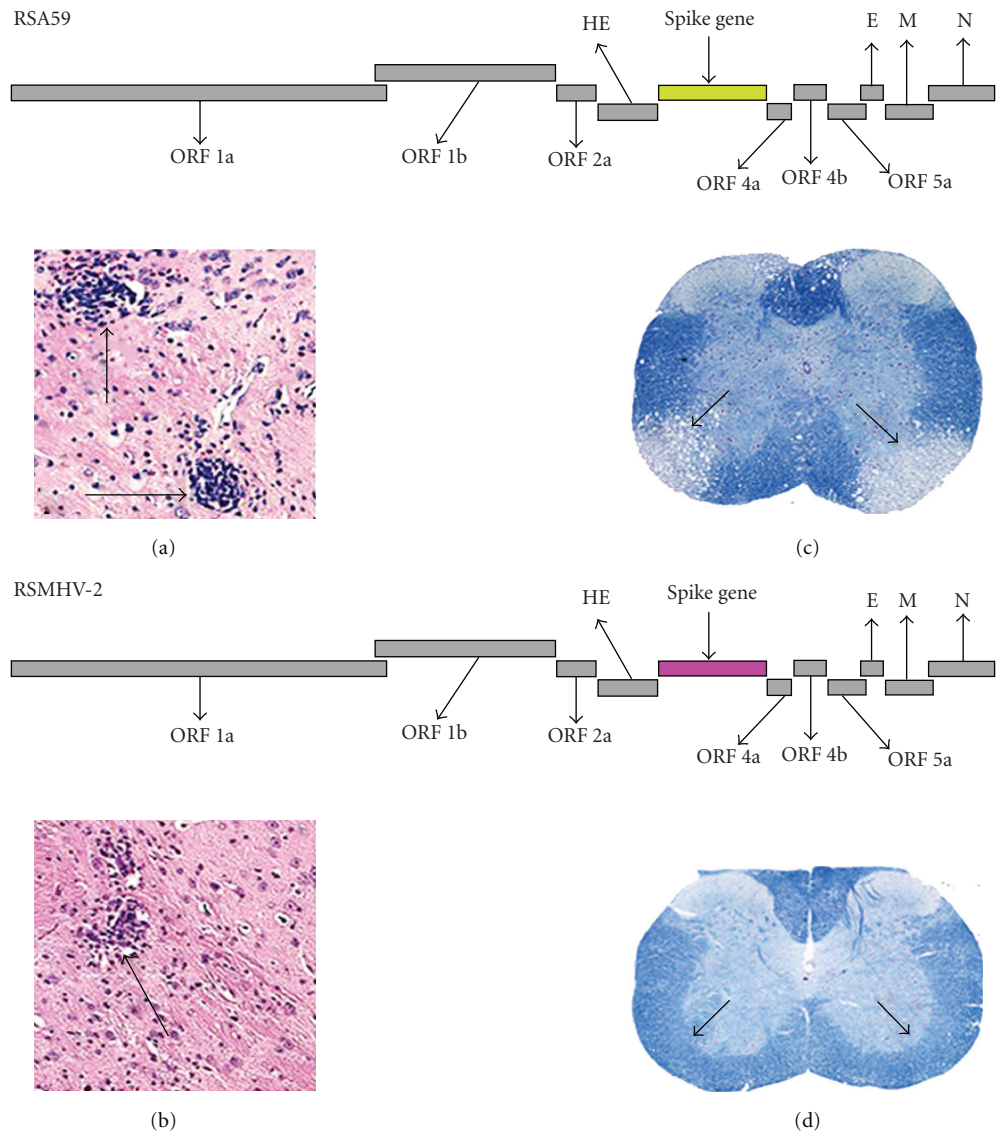



Figure 9: Comparative CNS pathology of mice infected with isogenic recombinant strains differs in the S gene. Schematic diagram of isogenic recombinant strains RSA59 (S gene denoted in green color) and RSMHV2 (S gene demoted in pink color). Encephalitis (a, b). Haematoxylin- and eosin stained brain sections from the basal fore brain of mice infected with either RSA59 (a) or RSMHV2 (b) at day 5 post infection. Arrows indicate regions of inflammation. Demyelination (c, d): spinal cord sections of mice infected with RSA59 (c) or RSMHV2 (d) at day 30 post infection were stained with Luxol fast blue. The arrows indicate a region of demyelination in (c) and normal myelinated areas of white matter in (d).

localization, the distribution of $RSMHV2_{EGFP}$ (NDM) strain is more localized in the brain parenchyma compared to $RSA59_{EGFP}$ (DM) strain.

*15.1. Colocalization of EGFP Positive Cells with Viral Antigen.* To confirm that EGFP positive cells were also positive for viral antigen, frozen sections were labeled with antinucleocapsid antiserum as primary antibody and Texas red goat antimouse IgG as secondary antibody. EGFP fluorescent was colocalized with viral antigen in both $RSA59_{EGFP}$-$RSMHV2_{EGFP}$ and-infected brain cortical sections (data not shown). There is a high degree of colocalization of EGFP and viral antigen in the majority of sections analyzed. Complete colocalization demonstrated that EGFP fluorescence can be used to detect viral antigen without performing any immunostaining.

*15.2. Comparative Neuropathology of EGFP Expressing DM and MDM Strains.* Pathology was also assessed in five to seven cross-sections of spinal cord from cervical, thoracic, and lumbar regions at day 7 (peak inflammation) and day 30 (peak demyelination) p.i. Demyelinating plaques were detected by LFB stains for myelin at day 7 p.i. in DM-infected mice (Figure 12(a)) and were quantified on a 0–3 scale [94] in four quadrants from two spinal cord levels for each mouse [116]. Day 30 p.i.-tissue sections from DM-infected mice

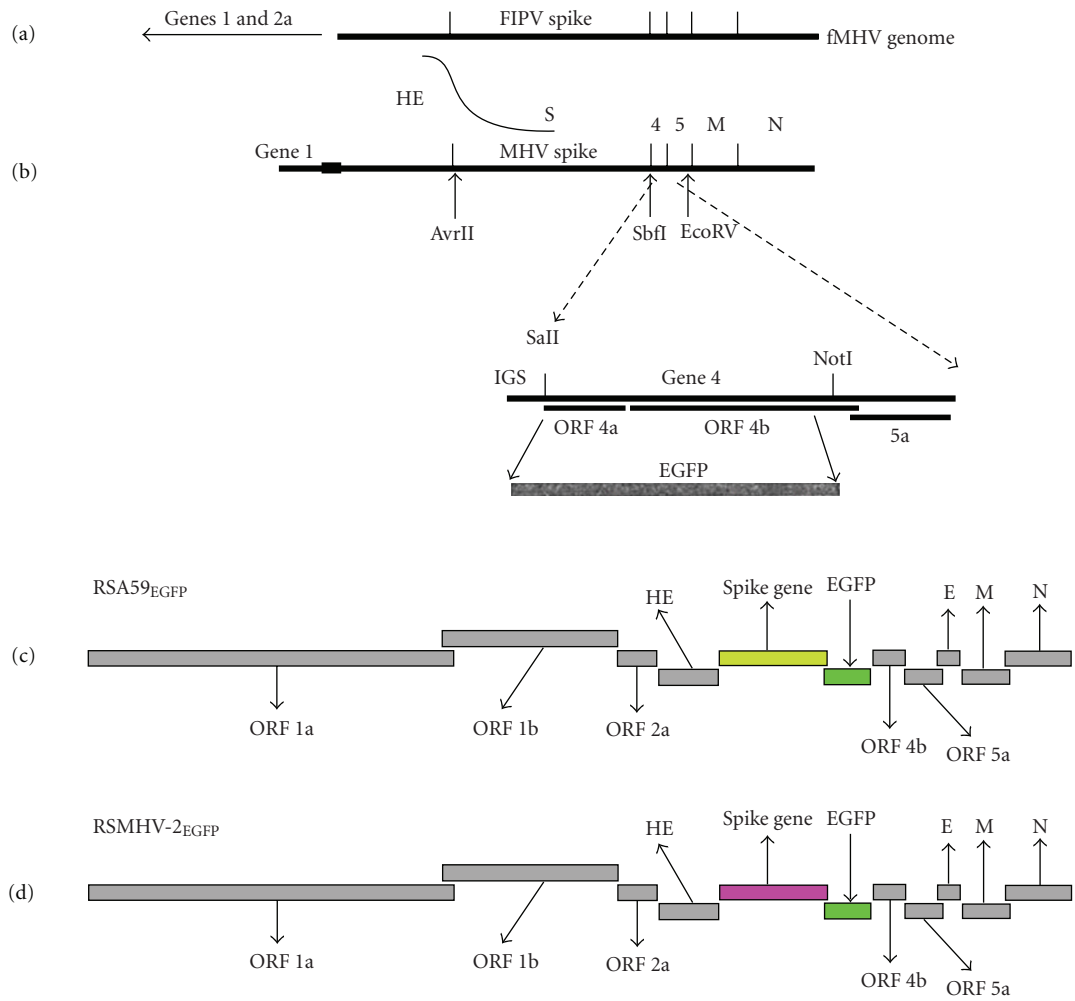
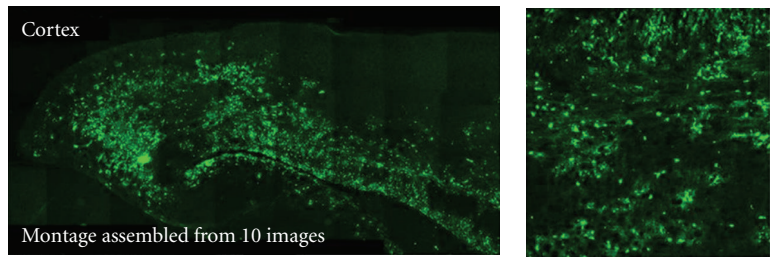



Figure 10: Schematic representation of targeted recombination to construct EGFP-expressing MHVs. The replacement of ORFs 4a and 4b with the EGFP gene was carried out by using recombinant technology, as described previously in [114]. (a) a schematic of the fMHV genome; it encodes the ectodomain of the feline infectious peritonitis spike in the background of the A59 genome. (b) shows the synthetic RNA transcribed from the vector $pMH54_{EGFP}$ (MHV-A59 S gene) or $pMHV\text{-}2_{EGFP}$ (MHV-2 S gene) [81, 114]. The curved line between the genome and the pMH54 RNA indicates the region in which the crossover must have occurred. The restriction sites relevant to the introduction of the EGFP gene are shown. The enlargement of the gene 4 region shows the modifications in which most of ORFs 4a and 4b are replaced by the enhanced green fluorescent protein (EGFP) gene. The IGS (intergenic sequence) is the site of initiation of transcription of mRNA 4. (c) and (d) show the resulting EGFP-expressing viruses: (c) $RSA59_{EGFP}$expressing the MHV-A59 S gene and (d) $RSMHV2_{EGFP}$ expressing the MHV-2 S gene. (adapted from the work of [114]).




(a) (b)

Figure 11: Detection of viral antigen spread by EGFP fluorescence. Mice ware sacrificed at day 5 p.i. Brain was removed and processed for frozen sections. A portion of sagittal brain sections was cut from the middle of the brain of infected mice. Frozen sections were processed for detection of viral antigen distribution by EGFP fluorescence. Bright EGFP fluorescence was spread throughout the brain section as showed by montage assembled from 10 serial images (a). Higher magnification image (b) from the same brain section shows the cellular distribution of EGFP fluorescence. (adapted from the wprk of [114]).

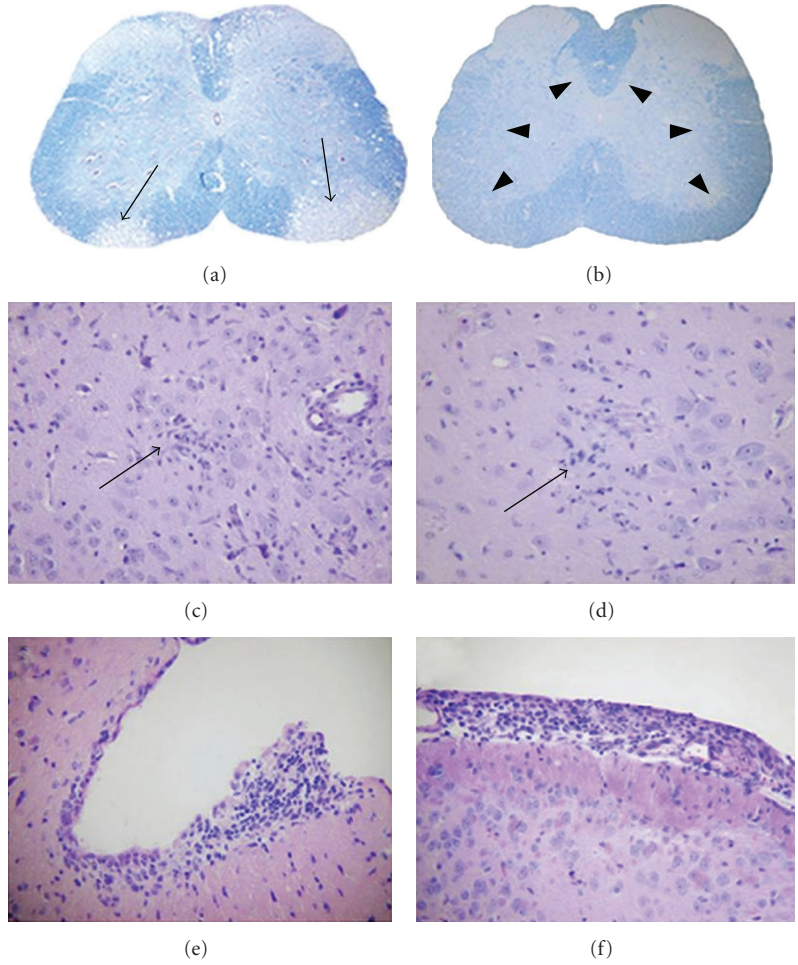



Figure 12: CNS pathology of EGFP expressing MHVs. (a, b) Demyelination: luxol fast blue-stained spinal cord sections of mice infected with $RSA59_{EGFP}$ (a) or $RSMHV2_{EGFP}$ (b), at day 30 post infection. Thin arrows indicate a region of demyelination, and the arrowheads indicate normal myelinated area of white matter. (c, d) Encephalitis: microglial nodules: Hematoxylin- and Eosin-stained sections from the basal forebrain of mice infected with EGFP tagged MHVs at day 7 post infection. Arrows indicate the microglial nodules (microglia with elongated nuclei) and lymphocytes in the vicinity of neurons in mice infected with $RSA59_{EGFP}$ (c) and $RSMHV2_{EGFP}$ (d). (e, f) Meningitis. Hematoxylin- and eosin-stained sections from the basal forebrain of mice infected with EGFP tagged MHVs at day 7 post infection: $RSA59_{EGFP}$ and $RSMHV2_{EGFP}$. In all cases, there is a brisk leptomeningeal lymphocytic inflammatory infiltrate. An original magnification for (a, b) is 20x and (c–f) is 200x. (adapted from the work of [116]).

showed a similar pattern of demyelination as on day 7, but the number and area of plaques ware larger [73]. NDM-infected mice did not exhibit any demyelination either at day 7 (Figure 12(b)) or at day 30 p.i. (data not shown) [73].

Though comparative histopathological studies demonstrated that $RSA59_{EGFP}$ could induce demyelination (Figure 12(a)) whereas $RSMHV2_{EGFP}$ cannot induce demyelination (Figure 12(b)), both $RSA59_{EGFP}$ and $RSMHV2_{EGFP}$ viruses produced meningoencephalitis (Figures 12(c)–12(f)). Brain pathology consisted of encephalitis, characterized by parenchymal lymphocytic infiltrates and microglial nodules with focal neuronophagia (Figures 12(c) and 12(d)). Brain sections from $RSA59_{EGFP}$ showed comparatively more parenchymal inflammation compared to $RSMHV2_{EGFP}$. Associated lymphocytic meningitis was also present in both the strains (Figures 12(e) and 12(f)).

*15.3. Significant Axonal Loss Occurs Concurrently with Demyelination Following $RSA59_{EGFP}$ Infection.* Though MHV-induced demyelination is characteristically described as sparing axons within areas of demyelination, axonal loss with demyelination in the optic nerve of MHV-A59- (parental DM) infected mice has been recently observed [94]. This led to hypothesize that DM MHV may induce axonal loss in the spinal cord. To examine this, serial sections of spinal cords from $RSA59_{EGFP}$- (DM) and $RSMHV2_{EGFP}$- (NDM)-infected mice were stained parallaly by LFB and Bielchowsky silver impregnation. DM-infected spinal cord sections at both day 7 and day 30 p.i. showed a significant decrease of axonal staining in demyelinating plaque (Figures 13(a) and 13(b)). No demyelination and no axonal loss ware evident in the NDM-infected mouse spinal cords either at day 7 (Figures 13(e) and 13(f)) or at day 30 p.i.

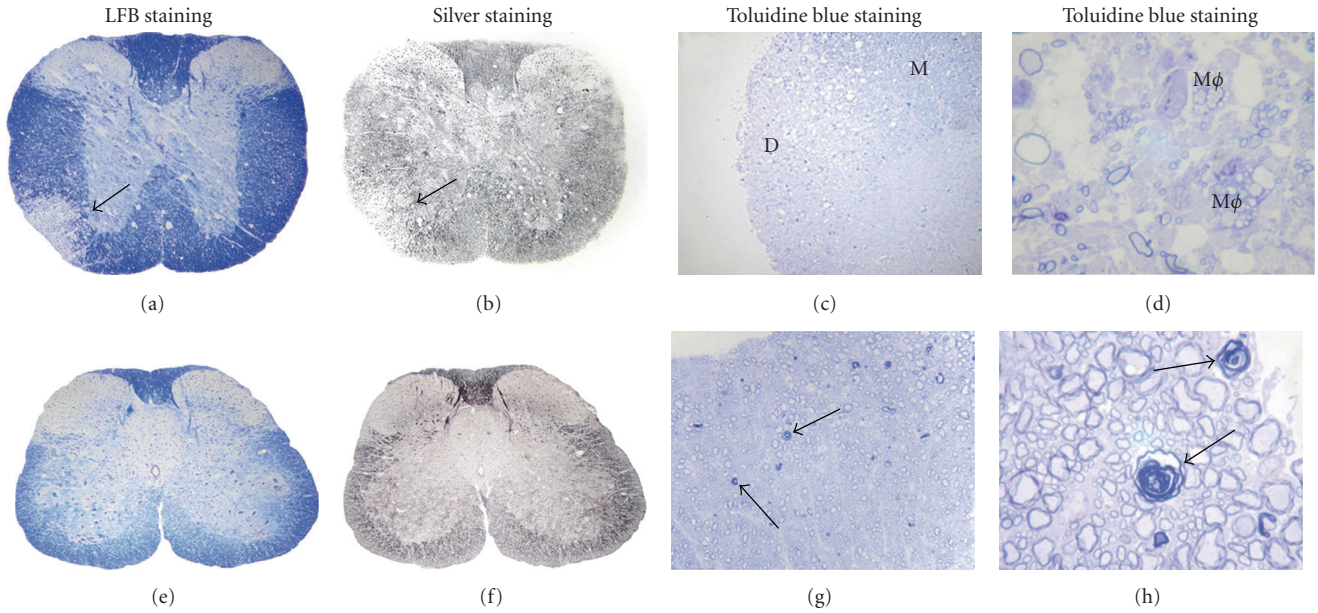



Figure 13: Demyelination and axonal loss in DM- and NDM-infected mouse spinal cord. Serial cross-sections (5 $\mu$m thick) from DM- and NDM-infected mouse spinal cord at day 7 p.i. were stained for myelin with LFB (a, e) or by Bielchowsky silver impregnation (b, f) or toluidine blue to delineate preservation of myelin, axons, and axon-myelin coherence following DM and NDM infection. (a) A large demyelinating plaque observed in DM-infected mouse spinal cord is shown (arrow indicates demyelinated area). (b) In an adjacent section, the same demyelinating plaque of DM-infected spinal cord showed loss of axons (arrow indicates area of axonal loss). (e) Normal myelin was observed in NDM-infected mouse spinal cord. (f) No axonal loss was observed in the NDM-infected mouse spinal cord. Spinal cord sections (1 $\mu$m thick) of mice sacrificed 30 days p.i. with DM and NDM were stained with toluidine blue (c, d, g, and h). Large demyelinated plaques were observed in DM-infected mouse spinal cord (C.D) . Myelinated spinal cord white matter region is marked (M), demyelinated region of spinal cord white matter (d), gray matter (g), macrophages (M$\phi$). In NDM-infected mouse spinal cord, myelin remains relatively preserved with rare examples of early axonal degeneration characterized by loss of the central axon and collapse of the myelin sheath (arrows) (g, h). Original magnification for (a, b, e, and f) is 40x. Original magnification for (c, g) is 100x and fot (d, h) is 1000x. (adapted from the work of [73]).

To further evaluate the loss and/or preservation of myelin in DM- and NDM-induced inflammatory plaques, semithin spinal cord sections cut at 1-micron intervals from five infected mice at day 30 p.i. were stained with toluidine blue. Control mock-infected mouse spinal cord was used to evaluate for background fixation and/or postfixation artifacts. DM-infected spinal cords showed significant myelin loss within plaques (Figure 13(c)). Moreover, high magnification images showed infiltrating macrophages filled with myelin debris (Figure 13(d)). In contrast, in NDM strain RSMHV2-infected spinal cord sections, myelin is almost completely preserved, with only rare examples of early axonal degeneration, characterized by loss of the central axon and collapse of the myelin sheath (Figures 13(g) and 13(h)). The presence of this focal axonal degeneration was not detectable in prior studies of paraffin-embedded tissues by silver impregnation.

*15.4. High-Resolution Electron Micrographic Analysis Confirms Axonal Loss Concurrent with Demyelination in RSA59$_{EGFP}$-Infected Mouse Spinal Cord.* To further characterize axonal pathology at the ultrastructural level, representative foci of demyelination and axonal injury were selected from toluidine blue-stained sections, and 600 Å ultrathin sections from Poly-Bed-embedded blocks were processed for TEM. High-resolution TEM images show a combination of axonal degeneration and demyelination. There are extensive axonal loss and many residual empty vacuoles corresponding to totally degenerated fibers. In addition, there are numerous hypomyelinated fibers and naked axons without any myelin sheath, indicative of a demyelinating process (Figures 14(b)–14(d)). These naked axons are fully intact with surrounding axolemmas and axoplasm with preserved microtubules and intermediate filaments (Figure 14(e)). In contrast, NDM-infected mouse spinal cords demonstrate no appreciable axonal loss and no features of demyelination. Specifically, there are no hypomyelinated fibers, no naked axons, no macrophages, and no evidence of macrophage-mediated myelin stripping. Instead, there are only rare examples of early axonal degeneration characterized by loss of the central axon and collapse of the myelin sheath (Figure 14(f)) as previously seen in toluidine blue-stained sections. Normal myelinated region is denoted in Figure 14(a).

*15.5. Macrophage-Mediated Myelin Stripping in DM-Infected Spinal Cord Section.* One mechanism of demyelination is revealed as macrophage-mediated myelin stripping. This is demonstrated in Figure 15 in which a macrophage is observed surrounding a myelinated axon. The myelin is unraveling, yet the axon is completely intact. At the inner

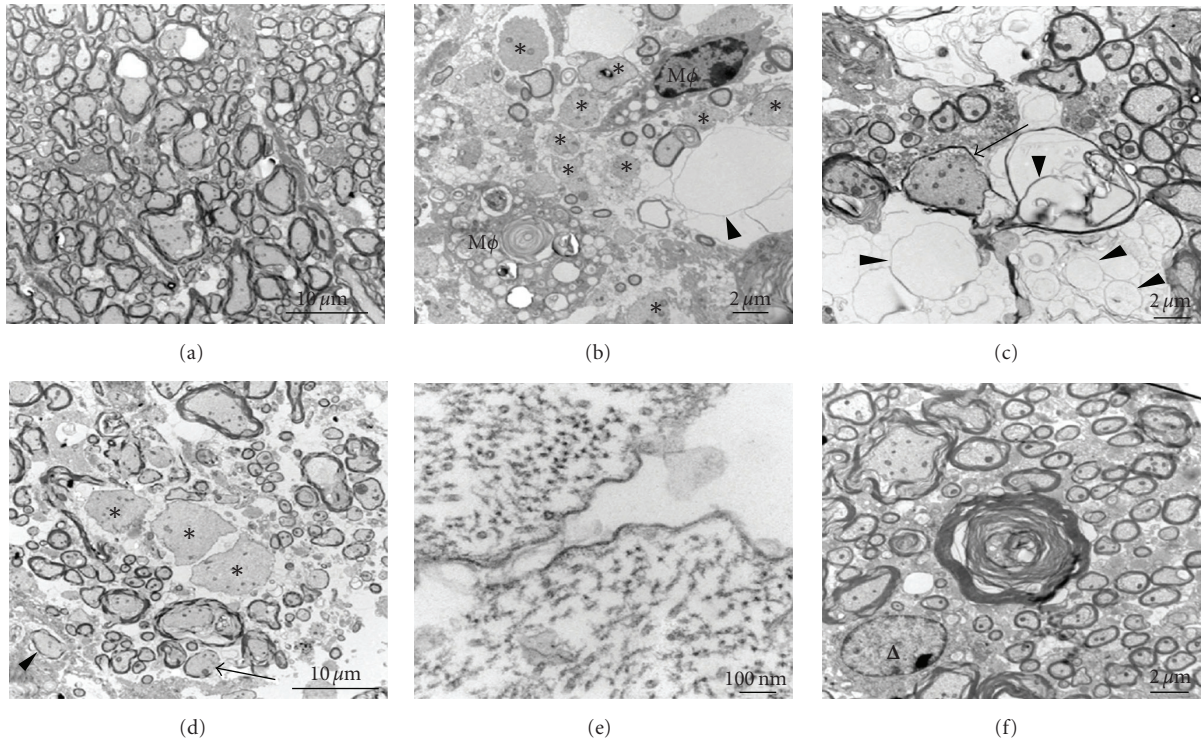



Figure 14: Electron micrographic analysis of demyelination and axonal loss in DM- and NDM-infected mouse spinal cord. Representative foci of demyelination and axonal injury were selected from the toluidine blue-stained sections shown in Figure 3 and processed for electron microscopy. (a) Normal morphology of myelinated axons was exhibited from a non plaque region adjacent to a demyelinated plaque of DM-infected mouse spinal cord. (b–d) Demyelinating plaque in DM-infected mouse spinal cord showed extensive loss of myelin, hypomyelination (arrows), complete axonal degeneration with only residual empty vacuoles (arrowheads), naked axons with no myelin sheath at all (*), and macrophages (M$\phi$). (e) A high-magnification image (120,000x) of a naked axon surrounded only by a single lipid bilayer shows preservation of cytoskeleton elements (microtubules and intermediate filaments). (f) In contrast, NDM-infected mouse spinal cords show only rare examples of early axonal degeneration. $\triangle$ Marks an oligodendrocyte nucleus. Such early axonal degeneration was not observed in mock-infected mice. Original magnifications: (a) 3000x, (b) 5000x, (c) 6000x, (d) 3000x, (e) 120,000x, and (f) 6000x. (Adapted from the work of [73]).

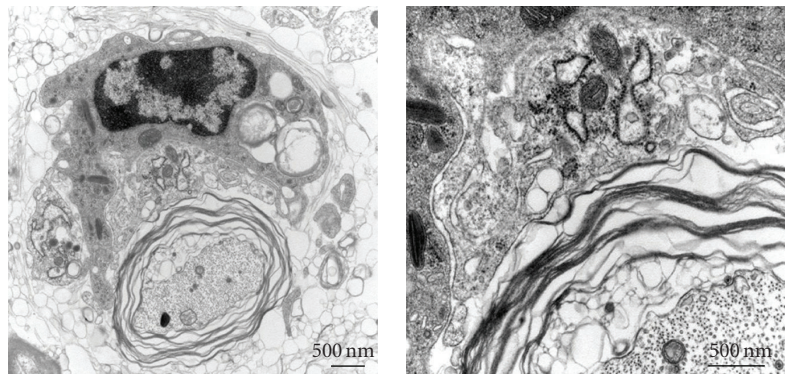



Figure 15: Macrophage-mediated myelin stripping in DM-induced demyelinating plaque. (a) A macrophage (upper portion of the figure) is observed surrounding an intact axon (lower portion of the figure) with uncompacted myelin. Myelin figures are observed within the cytoplasm of this macrophage indicative of prior engulfment of myelin. (b) Higher magnification demonstrates close apposition of the macrophage cell membrane and an outer layer of uncompacted myelin. The axoplasm and axolemma are intact and the adjacent myelin shows vesiculation indicative of myelin degeneration. Original magnification for (a) is 15,000x and for (b) is 40,000x. (adapted from the work of [73]).

border of the myelin sheath, multiple vacuoles are present as the entire myelin sheath is lifting off the axon. The macrophage cell membrane is in intimate contact with the outer portion of the myelin sheath as the macrophage strips away and engulfs the myelin sheath. Multiple vacuoles with myelin fragments are seen within the cytoplasm of the macrophage.

*15.6. Spinal Cord Inflammatory Plaques Contain Predominantly Macrophages.* To characterize inflammatory cells within spinal cord plaques, serial sections from DM- and NDM-infected mice were stained with anti-CD45 (LCA), anti-CD11b, or anti-CD3. LCA staining confirmed the presence of infiltrating inflammatory cells (Figures 16(c) and 16(d)). Interestingly, the distribution of $LCA^+$ cells was significantly different after infection by the two viral strains. At day 7 p.i., the majority of infected cells in $RSA59_{EGFP}$-infected spinal cord were localized to white matter with only occasional infiltration of gray matter (Figures 16(a) and 16(c)) whereas in $RSMHV2_{EGFP}$-infected spinal cord, $LCA^+$ cells were mainly restricted to gray matter (Figures 16(b) and 16(d)). CD11b staining demonstrated that the majority of $LCA^+$ cells in the spinal cord were $CD11b^+$ (Figures 16(e) and 16(f)). At day 30 p.i., $LCA^+$ cells were still present in abundance in demyelinating plaques produced following DM infection (Figures 16(g) and 16(i)), but in NDM-infected mice, no demyelinating plaques were present and very few $LCA^+$ cells were retained in the gray matter (Figures 16(h) and 16(j)). Immunohistochemical data demonstrate that in DM- and NDM-strain-infected spinal cord there is an increase of $LCA^+/CD11b^+$ cells; however, the distribution of these inflammatory cells is significantly different between the two strains.

*15.7. DM and NDM Strains Differ in Neuronal Intracellular Distribution of Viral Antigen.* To determine whether differential localization of viral antigen is responsible for the observed distribution of inflammatory cells, cross-sections from spinal cord of DM- and NDM-infected mice were examined. Since the DM and NDM viruses used in these studies express EGFP, viral distribution was assessed directly by fluorescence microscopy. On day 7 p.i., the DM fluorescence was mainly restricted to white matter (Figures 17(a) and 17(b)) with limited involvement of gray matter whereas NDM fluorescence was restricted to gray matter (Figures 17(c) and 17(d)). High-magnification fluorescence images in brain from day 7 p.i. demonstrate numerous neurons and their axons following DM infection (Figure 17(e)) whereas far fewer fluorescent neurons and axons are observed following NDM infection (Figure 17(f)). The ability of DM and NDM to replicate and spread in vitro was compared using primary hippocampal neuronal cultures and described in very recent studies . Immunostaining of MAP2b [117] and GFAP [118] demonstrated that cultures consisted primarily of neurons. At 12 hours intervals, from 0 to 72 hours p.i., cultures were monitored for viral antigen spread by fluorescence microscopy. At 24 hours p.i. several infected foci were observed in DM-infected cultures (Figure 17(g)) whereas, in NDM-infected cultures, very few discrete cells were positive for EGFP (data not shown). At 72 hours p.i., the average number of EGFP positive cells was significantly increased following infection with DM (Figure 17(h)). In contrast, NDM-infected cultures had viral infection restricted only to the initially infected neurons, with little or no spread to adjacent cells, consistent with in vivo studies (Figure 17(i)) [73].

To confirm the differential distribution of viral antigen in gray and white matter in very recent studies [116] cross-sections from several regions of all spinal cords were stained with viral antinucleocapsid antiserum. At day 5 post-infection viral antigens were mainly observed in the gray matter of the spinal cord in $RSA59_{EGFP}$- and $RSMHV2_{EGFP}$- infected mice some viral antigen was also detected in the white matter. At day 7, most of the viral antigen in $RSA59_{EGFP}$ was found in white matter whereas $RSMHV2_{EGFP}$ viral antigen remained mainly restricted to gray matter with occasional distribution to white matter. In $RSMHV2_{EGFP}$- infected mice, viral antigen was occasionally observed in the white matter, and in some spinal cord sections of $RSMHV2_{EGFP}$ viral antigen was detected at the gray-white matter junction as shown in very recent studies [116].

*15.8. DM and NDM Strains Exhibit Different Cellular Tropism.* The severity of tissue destruction in MHV infection is mediated by direct viral infection and immune-mediated destruction. The relative contributions of these two components differ depending on a number of virus and host factors including viral tropism, rate of viral spread, and specificity of the immune response. During acute infection, neurotropic and demyelinating strains (DM) of MHV infect a number of cell types in the CNS such as neurons, astrocytes, oligodendrocytes, and microglia [21, 70, 91, 118, 119]. Recently by using the S gene recombinant virus ($S_4$ R, containing S gene from a highly neurovirulent MHV-4 on the background of MHV-A59) *it has been* demonstrated that S gene-mediated neurovirulence of MHV is associated with extensive viral spread in the brain in both neurons and astrocytes [108]. Thus far it was not known whether NDM of MHV infects the same cell types as DS. The phenomenon of demyelination may be due to primary infection of a single cell type, or alternatively, infection of diverse CNS cell types leading to the final destruction of OLGs/myelin sheath and the axon.

To compare the cellular tropism of DM and NDM MHV double-label immunofluorescence on sagittal brain sections was performed [116]. EGFP fluorescence was used as a viral antigen marker, MAP2b as a neuron-specific marker, and GFAP as an astrocyte-specific marker. Sections were systematically scanned in a blinded fashion. Representative pictures of dual color fluorescence of viral antigen and MAP2b are shown in Figure 18. Visual microscopic observations show that $RSJHM_{EGFP}$ infects and spreads more in neurons in comparison to the other two strains. In $RSJHM_{EGFP}$- infected mice only a very small number of viral antigen positive cells were also positive for the astrocytic marker GFAP, whereas in $RSA59_{EGFP}$-infected mice, EGFP positive cells partially

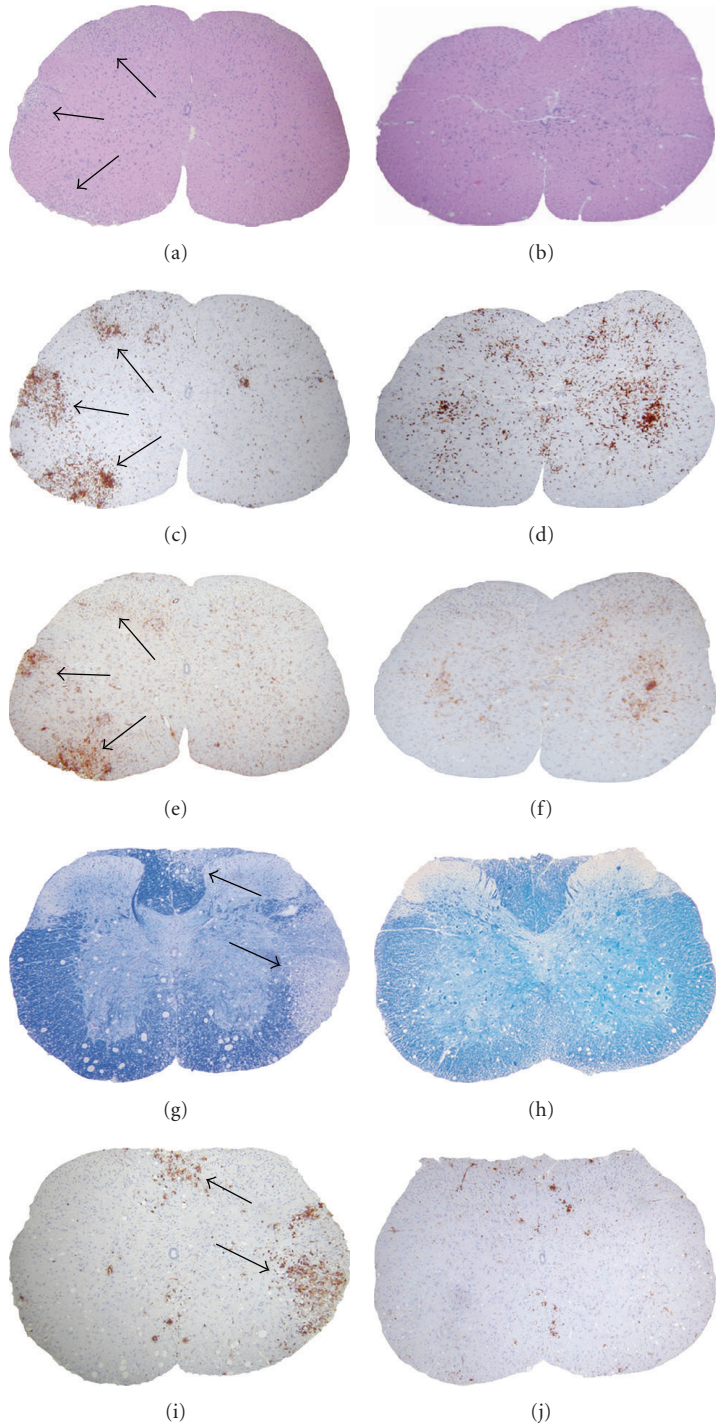



Figure 16: Distribution of inflammatory cells in the gray matter versus white matter in DM- and NDM-infected mouse spinal cord during acute infection. Serial cross sections (5 $\mu$m thick) from DM- and NDM-infected mouse spinal cords at day 7 p.i. were stained with H & E (a, b), LCA (c, d), or CD11b (e, f). Several inflammatory lesions (a) with infiltrating LCA$^+$ cells (c) were observed in RSA59$_{EGFP}$-infected mouse spinal cord (arrows indicate lesion areas in white matter). The majority of the LCA$^+$ inflammatory cells in the inflammatory plaques were positive for the microglia/macrophage marker CD11b (e). CD11b$^+$ cells in DM-infected mice are predominantly in the white matter. In NDM-infected mice no discrete inflammatory lesions were observed (b); however LCA$^+$ cells were observed (d) scattered throughout the gray matter. These cells were also strongly immunoreactive for CD11b (f). Most of the LCA/CD11b immunoreactive cells were restricted to gray matter with very little invasion of white matter. Serial cross sections (5 $\mu$m thick) from DM- or NDM-infected mouse spinal cords at day 30 p.i. were stained with LFB (g, h) or LCA (i, j). Large demyelinating lesions (g) with infiltrating LCA$^+$ cells (i) were observed in DM-infected mouse spinal cord white matter (arrows indicate lesion area) whereas normal myelin (h) was observed in NDM-infected mouse spinal cord with only rare scattered LCA$^+$ cells in the gray matter (j). Original magnification for (a–j) is 40x. (adapted from the work of [73]).

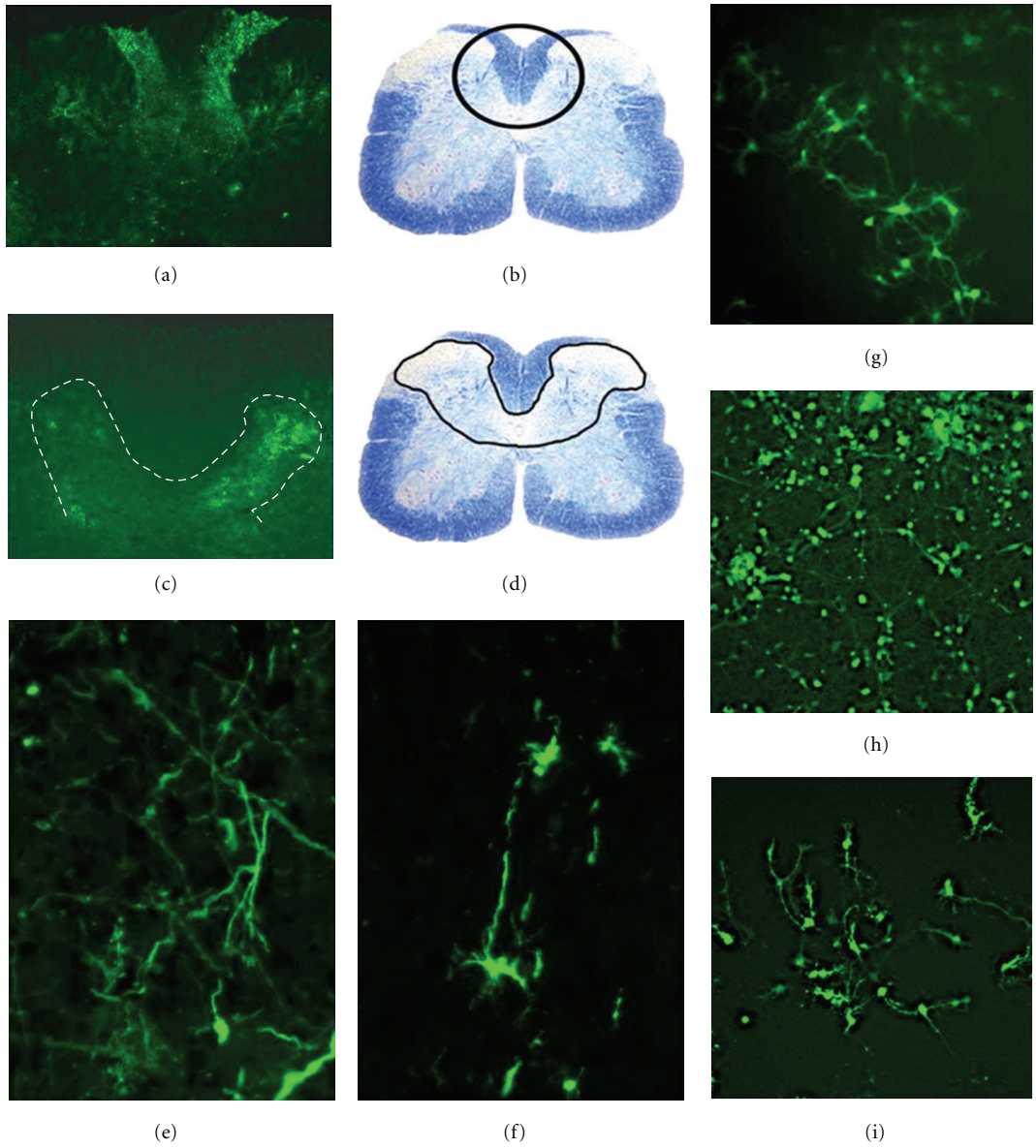



Figure 17: DM and NDM differ in their ability to translocate from gray matter to white matter during acute infection. DM- and NDM-infected mice were sacrificed at day 7 p.i.; brain and spinal cord tissues were harvested and processed for frozen sections. Cryostat sections were post fixed with 95% ice-cold ethanol for 20 minutes and observed by fluorescent microscopy for viral antigen (EGFP fluorescence). (a) In DM-infected spinal cord, viral antigen positive EGFP-expressing cells are present in both gray and white matters. (b) Area of EGFP positive cells from (a) is marked in a corresponding LFB-stained section. (c) In NDM-infected mice, EGFP positive cells with viral antigen are predominantly restricted to the gray matter. (d) Area of EGFP positive cells from (c) is marked in corresponding LFB-stained section. (e) Axonal distribution of the EGFP fluorescent viral antigen is evident in DM-infected brain. (f) NDM-infected neurons in the brain show considerably reduced axonal transport of viral antigen. (g–i) to further confirm the ability of DM and NDM to infect neurons and spread from neuron to neuron, 4-day-old primary hippocampal neuronal cultures were infected with DM and NDM at MOI of 2. Representative fields containing an equivalent density of cells are shown in each image. (g) At 24 hours p.i. DM infect a small percentage of the neurons in culture. (h) By 72 hours p.i. DM-infected neurons were observed to spread from one neuron to the next, as nearly all cells in the culture contained viral antigen despite initial infection of only a small percentage of cells. (i) NDM-infected neurons, on the other hand, demonstrate limited axonal transport efficiency at 72 hours p.i., with only a small number of cells containing a bright concentrated signal that is retained after primary infection. (adapted from the work of [73]).

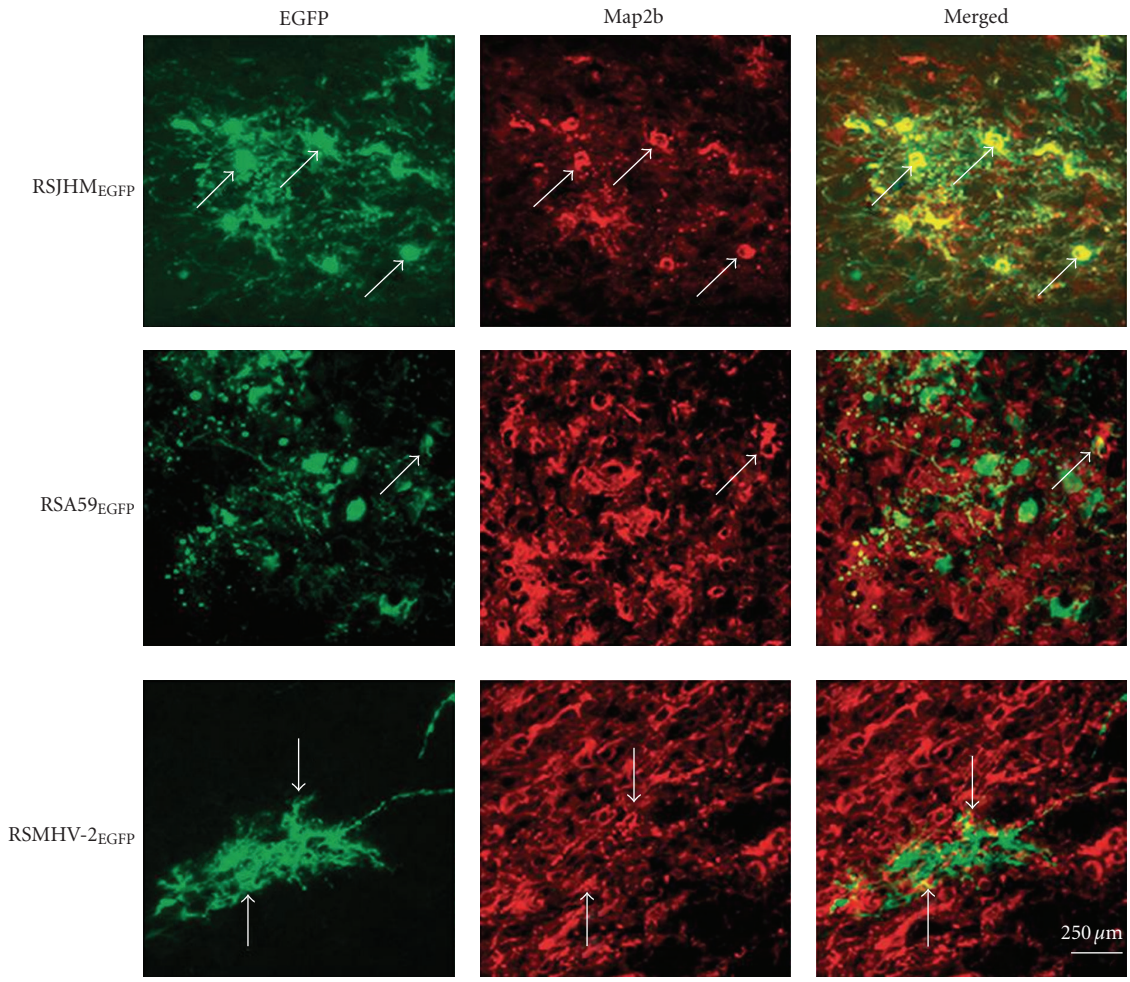



Figure 18: Identification of EGFP MHV-infected neurons in the brain at day 5 post infection. Mice were infected intracranially with RSJHM$_{EGFP}$, RSA59$_{EGFP}$, and RSMHV2$_{EGFP}$. Infected mice were sacrificed at day 5 post infection, and the brains were processed, sectioned and then labeled with anti-MAP2b as primary antiboelies and with Texas red goat anti-mouse IgG as secondary antibodies. EGFP fluorescence (green) was used to detect viral antigen-positive cells in the basal forebrain. Red fluorescence shows the corresponding labeling of MAP2b in neurons. Merged images show colocalization of MAP2b and EGFP positive cells. Arrows identify cells double positive for viral antigen and MAP2b. Bar = 250 microns. (Adapted from the work of [116]).

overlapped with GFAP staining (Figure 19). In addition, in RSA59$_{EGFP}$- infected mice EGFP positive cells were not only astrocytes but also other cell types. In contrast, in RSMHV2$_{EGFP}$-infected mice EGFP completely colocalized with GFAP (Figure 19).

Localization studies showed that MHV strains differ in their neurovirulence and ability to demyelinate and also differ in their ability to infect particular CNS cell types. RSJHM$_{EGFP}$ infects neurons with greater efficiency, and infection may spread more rapidly than in RSA59$_{EGFP}$, while RSMHV2$_{EGFP}$ infects neurons with limited efficiency. This is consistent with previous findings that the degree of neurovirulence is not only dependent on neuronal tropism but also on the number of infected neurons and spread of the infection through neurites [108]. Evidence from closely related strains of neurotropic viruses including HIV, TMEV, and reovirus supports the hypothesis that CNS cell tropism and spread in CNS cells play a major role in the distribution and type of CNS lesions [120, 121]. Studies with LCMV and HIV also suggest that virus strains that exhibit rapid spread are associated with increased immune-mediated pathology [122, 123]. Recently, it has been demonstrated that MHV spread to the spinal cord white matter occurs very rapidly, and protection from demyelination can be achieved by inhibiting this viral spread during the acute phase of infection by recruiting high numbers of MHV-specific CD8+ T cells [124]. Nondemyelinating strain is less able to infect neurons and spread through neurons; on the other hand, demyelinating strains are highly neuron tropic and spread rapidly through neurites. In contrast, it has been observed that a temperature-sensitive demyelinating mutant of JHM infects mainly nonneuronal cells, having

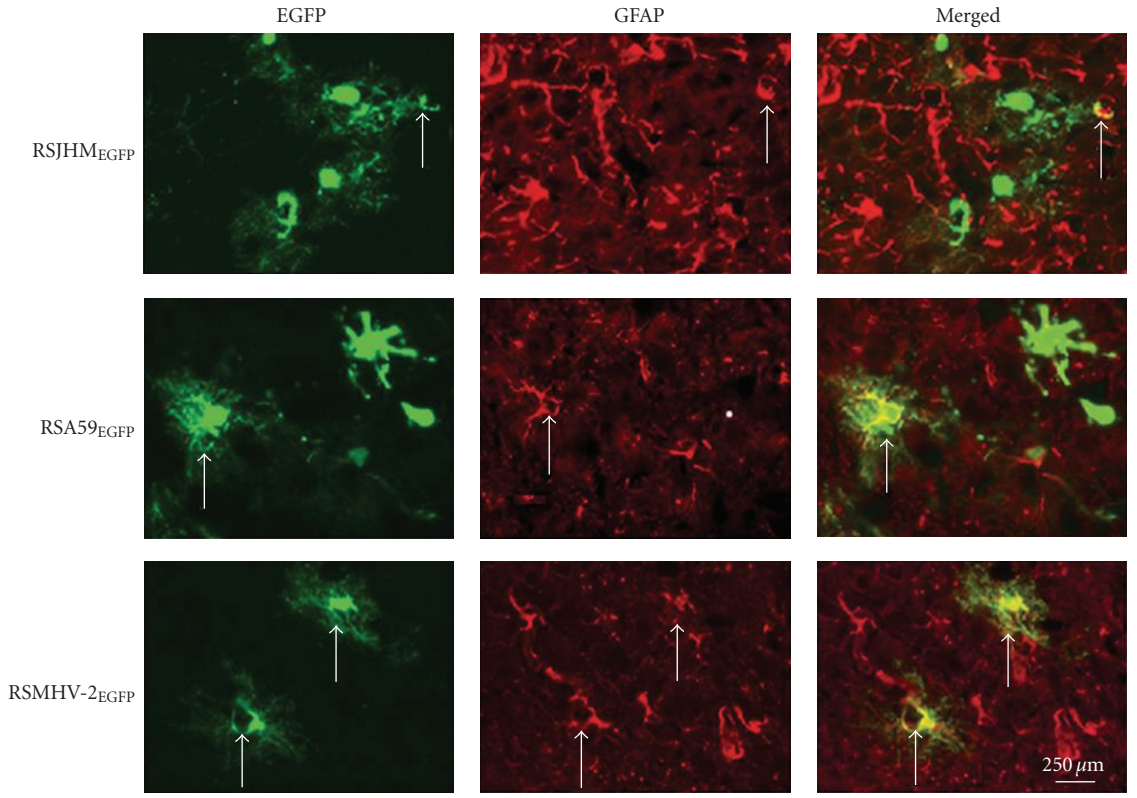



Figure 19: Colocalization of EGFP positive cells with astrocyte marker at day 5 post-infection. Mice were infected intracranially with RSJHM$_{EGFP}$, RSA59$_{EGFP}$ and RSMHV2$_{EGFP}$. Infected mice were sacrificed at day 5 post-infection; brains were processed, sectioned and then immune labeled with anti GFAP (astrocytic marker) antiserum, then stained with Texas red goat anti-mouse IgG as secondary antibodies. EGFP fluorescence was used to detect viral antigen positive cells; red fluorescence was used to detect GFAP in astrocytes. Merged images show colocalization of GFAP and EGFP positive cells. Arrows identify cells double positive for viral antigen and GFAP. Bar = 250 microns. *(Adapted from Das* [116]*).*

a strong tropism specifically for astrocytes and causes white matter lesions. Similarly, it has been demonstrated that a monoclonal antibody selected JHM variant, a S protein mutant, infects the glial cells predominantly in the CNS, causing subacute demyelination. Interestingly, the neurotropic and nondemyelinating MHV3 strain, which has an in vitro tropism for neurons, ependymal cells, and meningeal cells but not for astrocytes and oligodendrocytes, can induce initial ependymitis, meningitis, and encephalitis in the absence of white matter lesions. Our in vivo data demonstrate that the highly neurovirulent strain RSJHM$_{EGFP}$ has very little tropism for astrocytes. RSA59$_{EGFP}$ and RSMHV2$_{EGFP}$ infect astrocytes with similar efficiency. As demyelinating and nondemyelinating strains produce similar infections in astrocytes, it appears that astrocytic infection during the acute stage alone may not determine the onset of demyelination. Our observations suggest that astrocyte tropism may be a relevant factor in persistent infection but direct astrocytic infection alone may not be sufficient to induce demyelination. This study highlights the important role of neural cell tropism of viral antigen in MHV-induced demyelination in the CNS.

## 16. Neural Cell-Specific Viral RNA Persistence in Induction of Demyelination and Axonal Loss

The development of chronic demyelination involves replication and spread of viral antigen in the spinal cord during the acute stage of infection and the persistence of viral RNA in white matter. This is substantiated by current observations on viral spread of NDM MHV in the spinal cord, where viral antigen during the acute phase of infection is mainly restricted to gray matter. In contrast, viral antigen from demyelinating strains spreads from gray to white matter during the acute stage of infection [73, 116]. Specifically, after intracranial infection and replication, viral antigens from the demyelinating strains RSJHM$_{EGFP}$ and RSA59$_{EGFP}$ are mostly seen in spinal cord gray matter at day 5 post infection and then in the white matter at day 7 post infection. This finding establishes that demyelinating and nondemyelinating strains differ in their viral antigen distribution in gray matter and white matter during acute infection. Viral persistence observed previously in RSMHV2-infected mouse spinal cord during the chronic phase of infection may be due

to the presence of viral RNA in gray matter. Viral RNA for demyelinating strains is known to persist mainly in the white matter. Due to lack of viral RNA persistence in the white matter, essential for the induction of demyelination during chronic infection, RSMHV2 is unable to induce demyelination. It is possible that cell-specific viral RNA persistence is necessary for demyelination. These combined observations reinforce the importance of glial cell infection in the onset ofdemyelination.

## 17. Putative Mechanistic Aspect of DM Strain-Induced Demyelination and Axonal Loss

Based on experimental evidences it can also be argued that the spread of viral antigen from neuron to neuron and from neuron to glial cells plays a critical role in the induction of chronic stage demyelination. Both DM and NDM strains infect neurons; however, these strains differ in their capacity to translocate to white matter as determined by fluorescence of EGFP tagged viral particles. The current experiments indicate that axonal transport of viral particles is an important mechanism mediating not only the extent of axonal damage but also the subsequent induction of demyelination in the spinal cord. DM strain shows white matter involvement early (by day 7 p.i.) and at later time points that gray matter involvement is essentially absent in the spinal cord, with only minimal involvement in the brain. This observation further supports earlier findings that DM and NDM strains differ in their viral antigen distribution during acute infection in the spinal cord [116]. Evaluation of axonal loss and demyelination in the spinal cord, where there is clear separation of gray matter and large tracts of white matter, demonstrated that DM MHV infection begins in the neuronal cell body and propagates centripetally to the axon [73] which subsequently induces axonal degeneration and demyelination. NDM strains were unable to propagate from gray to white matter and as a result and were unable to induce demyelination.

The migration and activation of numerous CD11b+ macrophages/microglia to the white matter following viral spread of DM MHV [73] suggests that recruitment of these cells mediates demyelination. During DM strain chronic infection at day 30 p.i., macrophages/microglia were still present within areas of demyelination whereas macrophages/microglia and viral antigen were cleared from the gray matter following NDM strain infection.

One plausible explanation is that MHV spreads intra-axonally in an anterograde manner within gray matter and when it reaches the white matter, viral particles are able to infect oligodendrocytes via direct cell-cell contact. A less likely possibility is that infection proceeds indirectly from neurons to oligodendrocytes by intermediary cells such astrocytes, microglia, or endothelial cells. Regardless of the mechanism of viral spread, ultimately, macrophages and microglia are recruited to the areas of infection. In this way, MHV infection triggers axonal loss and macrophage-mediated demyelination. However, we have also observed in NDM strain infection, that axonal degeneration and myelin disruption can occur in the absence of macrophages. Therefore, MHV infection can both directly and indirectly cause axonal loss and demyelination. It may be that the failure of NDM strain to trigger macrophage-mediated damage is entirely a function of transport failure to the white matter.

Although there are no experimental data to demonstrate this, it can be suggested, based on our current neural cell tropism studies, that neuroinvasion by MHV may require entry into the nerve endings, transport to the cell body, replication in the cell body, axonal transport to the synapse, and transneuronal viral spread. Similar mechanisms of axonal transport of virus particles have been observed for the alpha herpesviruses, Herpes simplex virus and pseudorabies virus [125, 126]. Furthermore, Theiler's murine encephalitis virus (TMEV), a nonenveloped virus that, like MHV, causes chronic demyelination in mice, is able to traffic from the axon into the surrounding myelin in the absence of cell lysis [127]. Thus, by a mechanism involving transneuronal spread, MHV may gain access to synaptically linked neuronal circuits and glial cells. It is not clear, however, which type (s) of glial cells must be infected in order to promote the development of MHV-induced chronic demyelinating disease.

Available studies focus mainly on neurons and astrocytes, as they represent the majority of infected cells during the acute stage of MHV infection, but oligodendrocytes cannot be disregarded. It has been previously demonstrated that MHV can infect oligodendrocytes [128], but the direct cytolytic effect of MHV on oligodendrocytes is unclear [129, 130]. Direct viral infection of oligodendrocytes may contribute to MHV-induced demyelination, as observed in another human demyelinating disease, progressive multifocal leukoencephalopathy (PML), which is caused by JC virus infection of oligodendrocytes [131, 132].

## 18. Putative Molecular Mechanisms of Demyelination and Axonal Loss

The current studies further demonstrate that the molecular mechanisms underlying axonal damage are mediated by the S glycoprotein. The recombinant DM and NDM viruses used are isogenic on a background of the DM MHV-A59 strain and differ only by the virus-host attachment S glycoprotein. In vivo, the S protein host receptor is carcinoembryonic antigen (CEA), which functions as an intercellular adhesion molecule [56, 133, 134]. However, there is evidence that other viral entry host factors may also be permissive for viral infection [135]. Evidently, the difference in the S proteins between the DM and NDM strains does not impair host-receptor interactions or viral entry since both strains effectively cause encephalitis following transcranial inoculation. The S protein does, however, appear to play a critical role in axonal transport, with a lack of viral antigen spread and subsequent inflammation extending into spinal cord white matter following infection with the NDM strain in contrast to extensive white matter involvement secondary to DM strain infection being observed. As mentioned earlier, MHV S is synthesized as a 180kDa glycosylated precursor

that is posttranslationally cleaved into two 90kDa subunits, S1 and S2 [136], with a receptor-binding domain in the S1 subunit [137] that is responsible for the initial attachment of MHV to cell surface receptors. This binding event triggers a conformational change in S that allows S2 to initiate fusion of the virus and host membranes [138–140]. A candidate fusion peptide domain has been identified within S2 [141]; however, the actual fusion peptide for the MHV S has not been definitively identified. MHV-A59 and MHV-2 S proteins have 82% amino acid sequence identity and 94% similarity, with the S2 domain relatively more conserved and S1 more variable [93]. They also differ in their cleavage signal site whereby MHV-A59 S is cleaved posttranslationally into S1 and S2 subunits, but MHV-2 S protein is not cleaved and unable to cause fusion in vivo and in vitro. Thus, variable regions of the S1 domain or differences in the cleavage signal site between DM and NDM strains are candidate domains that may potentially explain the differential axonal transport and demyelination observed in our studies and need to be evaluated further in future studies.

Genes other than the S sometimes referred to as "background genes" also influence pathogenesis. In addition to structural proteins and replicase, all MHVs encode small unique proteins with unknown functions (ORF2a, 4, and 5a) [142]. The proteins encoded in these three ORFs appear to be nonessential for replication. It is instructive to note that, apart from the molecular role of the S gene in MHV pathogenesis, several additional genes have also been implicated in the disease etiology. Asides from their structural roles, N and M proteins are believed to function in host interactions. In porcine coronavirus, TGEV, mutations in the M protein ectodomain that impair N-glycosylation decrease the interferogenic activity and antibodies directed to the TGEV M ectodomain block IFN-$\gamma$ induction [143]. For MHV, the glycosylation state of M protein may effect the ability to induce IFN$\gamma$ and also to replicate in the liver [144]. In measles infection, a defective M transmembrane protein has been related to the difference in the ability of the virus to persist in the CNS and cause subacute sclerosing panencephalitis (SSPE) [145]. The N protein has been implicated in MHV-induced hepatitis [146, 147]. In addition, the replicase and other non structural proteins could affect tropism and pathogenesis by influencing the rate of viral replication perhaps by interactions with cell type specific factors or with elements of the immune response [148].

## 19. Conclusion

Current available studies demonstrate that the mechanisms of white matter injury in this model of CNS demyelinating disease are due to a combination of both axonal injury/loss and myelin damage. In DM infection, there is concomitant axonal loss and demyelination, and at least some demyelination is due to macrophage-mediated myelin stripping. One can hypothesize that axonal degeneration follows in this immune-mediated pathogenesis. Partial axonal loss also appears to occur secondary to direct viral-induced cytopathic effects, a non-immune-mediated mechanism. Following NDM infection, although relatively rare, myelinated fibers in the spinal cord white matter exhibit early axonal degeneration in the absence of any associated inflammation. Therefore, MHV infection exhibits diverse mechanisms of axonal loss and demyelination that are both immune and nonimmune mediated. While the underlying mechanisms leading to the development of MS in patients are not known, numerous studies have provided evidence to suggest that a viral infection may trigger this autoimmune demyelinating disease. The critical role of the S glycoprotein in viral transport and subsequent axonal and myelin damage demonstrated in the current studies suggests that targeted disruption of the S glycoprotein-host interaction has the potential to prevent the onset or progression of demyelination. Future experiments need to be geared toward identifying how the S proteins interact with the axonal transport system. Clues may be provided by other human viral neurologic infections, including those caused by such diverse viruses as Herpes Simplex and Rabies, as they take advantage of the axonal transport system in order to maximize spread and neurovirulence. Experimental mouse model provides an excellent means by which the particular host-virus interactions responsible for successful axonal transport can be dissected and clarified.

## Acknowledgments

Viral-induced experimental animal model to understand the mechanism of demyelination research was supported by National Multiple Sclerosis Society, M. E. Groff Surgical Medical Research and Education Charitable Trust, and Lindback Foundation Career Enhancement Award. The success of this work has largely depended on contribution and devotion of many researchers whose names are listed in the references. Dr. Lawrence. C Kenyon is acknowledged for the pathological data analysis and Mr. Ryan Marek for image processing.

## References

[1] "National Multiple Sclerosis Society-About research," June 2004, http://www.nationalmssociety.org/research-factSheet.asp.

[2] D. E. McFarlin and H. F. McFarland, "Multiple sclerosis (first of two parts)," *The New England Journal of Medicine*, vol. 307, no. 19, pp. 1183–1188, 1982.

[3] D. E. McFarlin and H. F. McFarland, "Multiple sclerosis. (second of two parts)," *The New England Journal of Medicine*, vol. 307, no. 20, pp. 1246–1251, 1982.

[4] B. Ferguson, M. K. Matyszak, M. M. Esiri, and V. H. Perry, "Axonal damage in acute multiple sclerosis lesions," *Brain*, vol. 120, no. 3, pp. 393–399, 1997.

[5] B. D. Trapp, J. Peterson, R. M. Ransohoff, R. Rudick, S. Mork, and L. Bo, "Axonal transection in the lesions of multiple sclerosis," *The New England Journal of Medicine*, vol. 338, no. 5, pp. 278–285, 1998.

[6] M. A. Hern'n, S. M. Zhang, L. Lipworth, M. J. Olek, and A. Ascherio, "Multiple sclerosis and age at infection with common viruses," *Epidemiology*, vol. 12, no. 3, pp. 301–306, 2001.

[7] B. Hemmer, J. J. Archelos, and H.-P. Hartung, "New concepts in the immunopathogenesis of multiple sclerosis," *Nature Reviews Neuroscience*, vol. 3, no. 4, pp. 291–301, 2002.

[8] D. H. Gilden, "Infectious causes of multiple sclerosis," *Lancet Neurology*, vol. 4, no. 3, pp. 195–202, 2005.

[9] J. H. Connolly, I. V. Allen, L. J. Hurwitz, and J. H. Millar, "Measles-virus antibody and antigen in subacute sclerosing panencephalitis," *Lancet*, vol. 1, no. 7489, pp. 542–544, 1967.

[10] K. G. Porter, D. G. Sinnamon, and R. R. Gillies, "Cryptococcus neoformans specific oligoclonal immunoglobulins in cerebrospinal fluid in cryptococcal meningitis," *Lancet*, vol. 1, no. 8024, p. 1262, 1977.

[11] R. T. Johnson, "The virology of demyelinating diseases," *Annals of Neurology*, vol. 36, pp. S54–S60, 1994.

[12] W. A. Sibley, C. R. Bamford, and K. Clark, "Clinical viral infections and multiple sclerosis," *Lancet*, vol. 1, no. 8441, pp. 1313–1315, 1985.

[13] P. B. Challoner, K. T. Smith, J. D. Parker, et al., "Plaque-associated expression of human herpesvirus 6 in multiple sclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 16, pp. 7440–7444, 1995.

[14] F. G. A. Moore and C. Wolfson, "Human herpes virus 6 and multiple sclerosis," *Acta Neurologica Scandinavica*, vol. 106, no. 2, pp. 63–83, 2002.

[15] S. S. Soldan, T. P. Leist, K. N. Juhng, H. F. McFarland, and S. Jacobson, "Increased lymphoproliferative response to human herpesvirus type 6A variant in multiple sclerosis patients," *Annals of Neurology*, vol. 47, no. 3, pp. 306–313, 2000.

[16] K.-P. Wandinger, W. Jabs, A. Siekhaus, et al., "Association between clinical disease activity and Epstein-Barr virus reactivation in MS," *Neurology*, vol. 55, no. 2, pp. 178–184, 2000.

[17] C. N. Martyn, M. Cruddas, and D. A. S. Compston, "Symptomatic Epstein-Barr virus infection and multiple sclerosis," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 56, no. 2, pp. 167–168, 1993.

[18] M. C. Dal Canto, B. S. Kim, S. D. Miller, and R. W. Melvold, "Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination: a model for human multiple sclerosis," *Methods*, vol. 10, no. 3, pp. 453–461, 1996.

[19] E. L. Oleszak, J. R. Chang, H. Friedman, C. D. Katsetos, and C. D. Platsoucas, "Theiler's virus infection: a model for multiple sclerosis," *Clinical Microbiology Reviews*, vol. 17, no. 1, pp. 174–207, 2004.

[20] J. J. Houtman and J. O. Fleming, "Pathogenesis of mouse hepatitis virus-induced demyelination," *Journal of NeuroVirology*, vol. 2, no. 6, pp. 361–376, 1996.

[21] E. Lavi, D. H. Gilden, and Z. Wroblewska, "Experimental demyelination produced by the A59 strain of mouse hepatitis virus," *Neurology*, vol. 34, no. 5, pp. 597–603, 1984.

[22] S. A. Stohlman and L. P. Weiner, "Chronic central nervous system demyelination in mice after JHM virus infection," *Neurology*, vol. 31, no. 1, pp. 38–44, 1981.

[23] L. P. Weiner, "Pathogenesis of demyelination induced by a mouse hepatitis," *Archives of Neurology*, vol. 28, no. 5, pp. 298–303, 1973.

[24] E. Lavi, D. H. Gilden, M. K. Highkin, and S. R. Weiss, "Persistence of mouse hepatitis virus A59 RNA in a slow virus demyelinating infection in mice as detected by in situ hybridization," *Journal of Virology*, vol. 51, no. 2, pp. 563–566, 1984.

[25] S. Perlman, G. Jacobson, A. L. Olson, and A. Afifi, "Identification of the spinal cord as a major site of persistence during chronic infection with a murine coronavirus," *Virology*, vol. 175, no. 2, pp. 418–426, 1990.

[26] J. L. Gombold and S. R. Weiss, "Mouse Hepatitis Virus A59 increases steady-state levels of MHC mRNAs in primary glial cell cultures and in the murine Central Nervous System," *Microbial Pathogenesis*, vol. 13, no. 6, pp. 493–505, 1992.

[27] E. Lavi, A. Suzumura, L. A. Lampson, et al., "Expression of MHC class I genes in mouse hepatitis virus (MHV-A59) infection and in multiple sclerosis," *Advances in Experimental Medicine and Biology*, vol. 218, pp. 219–222, 1987.

[28] E. Lavi, A. Suzumura, E. M. Murray, D. H. Silberberg, and S. R. Weiss, "Induction of MHC class I antigens on glial cells is dependent on persistent mouse hepatitis virus infection," *Journal of Neuroimmunology*, vol. 22, no. 2, pp. 107–111, 1989.

[29] A. Suzumura, E. Lavi, S. R. Weiss, and D. H. Silberberg, "Coronavirus infection induces H-2 antigen expression on oligodendrocytes and astrocytes," *Science*, vol. 232, no. 4753, pp. 991–993, 1986.

[30] A. Suzumura, E. Lavi, S. Bhat, D. Murasko, S. R. Weiss, and D. H. Silberberg, "Induction of glial cell MHC antigen expression in neurotropic coronavirus infections. Characterization of the H-2-inducing soluble factor elaborated by infected brain cells," *Journal of Immunology*, vol. 140, no. 6, pp. 2068–2072, 1988.

[31] A. I. Kaplin, C. Krishnan, D. M. Deshpande, C. A. Pardo, and D. A. Kerr, "Diagnosis and management of acute myelopathies," *Neurologist*, vol. 11, no. 1, pp. 2–18, 2005.

[32] I. J. Koralnik, "New insights into progressive multifocal leukoencephalopathy," *Current Opinion in Neurology*, vol. 17, no. 3, pp. 365–370, 2004.

[33] M. C. Dal Canto and S. G. Rabinowitz, "Experimental models of virus-induced demyelination of the central nervous system," *Annals of Neurology*, vol. 11, no. 2, pp. 109–127, 1982.

[34] S. D. Miller, C. L. Vanderlugt, W. S. Begolka, et al., "Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading," *Nature Medicine*, vol. 3, no. 10, pp. 1133–1136, 1997.

[35] M. Rodriguez and S. Sriram, "Successful therapy of Theiler's virus-induced demyelination (DA strain) with monoclonal anti-Lyt-2 antibody," *Journal of Immunology*, vol. 140, no. 9, pp. 2950–2955, 1988.

[36] M. Yamada, A. Zurbriggen, and R. S. Fujinami, "Monoclonal antibody to Theiler's murine encephalomyelitis virus defines a determinant on myelin and oligodendrocytes, and augments demyelination in experimental allergic encephalomyelitis," *Journal of Experimental Medicine*, vol. 171, no. 6, pp. 1893–1907, 1990.

[37] I. Tsunoda, Y. Iwasaki, H. Terunuma, K. Sako, and Y. Ohara, "A comparative study of acute and chronic diseases induced by two subgroups of Theiler's murine encephalomyelitis virus," *Acta Neuropathologica*, vol. 91, no. 6, pp. 595–602, 1996.

[38] C. P. Rossi, M. Delcroix, I. Huitinga, et al., "Role of macrophages during Theiler's virus infection," *Journal of Virology*, vol. 71, no. 4, pp. 3336–3340, 1997.

[39] J. S. Haring, L. L. Pewe, and S. Perlman, "Bystander CD8 T cell-mediated demyelination after viral infection of the central nervous system," *Journal of Immunology*, vol. 169, no. 3, pp. 1550–1555, 2002.

[40] F.-I. Wang, S. A. Stohlman, and J. O. Fleming, "Demyelination induced by murine hepatitis virus JHM strian (MHV-4) is immunologically mediated," *Journal of Neuroimmunology*, vol. 30, no. 1, pp. 31–41, 1990.

[41] G. F. Wu, A. A. Dandekar, L. Pewe, and S. Perlman, "CD4 and CD8 T cells have redundant but not identical roles in virus- induced demyelination," *Journal of Immunology*, vol. 165, no. 4, pp. 2278–2286, 2000.

[42] A. E. Matthews, E. Lavi, S. R. Weiss, and Y. Paterson, "Neither B cells nor T cells are required for CNS demyelination in mice persistently infected with MHV-A59," *Journal of NeuroVirology*, vol. 8, no. 3, pp. 257–264, 2002.

[43] R. M. Sutherland, M.-M. Chua, E. Lavi, S. R. Weiss, and Y. Paterson, "$CD4^+$ and $CD8^+$ T cells are not major effectors of mouse hepatitis virus A59-induced demyelinating disease," *Journal of NeuroVirology*, vol. 3, no. 3, pp. 225–228, 1997.

[44] K. McIntosh, R. K. Chao, H. E. Krause, R. Wasil, H. E. Mocega, and M. A. Mufson, "Coronavirus infection in acute lower respiratory tract disease of infants," *The Journal of Infectious Diseases*, vol. 130, p. 502, 1974.

[45] M. M. Lai and D. Cavanagh, "The molecular biology of coronaviruses," *Advances in Virus Research*, vol. 48, pp. 1–100, 1997.

[46] J. M. Gonzalez, P. Gomez-Puertas, D. Cavanagh, A. E. Gorbalenya, and L. Enjuanes, "A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae," *Archives of Virology*, vol. 148, no. 11, pp. 2207–2235, 2003.

[47] J. D. Sarma, L. Fu, S. T. Hingley, and E. Lavi, "Mouse hepatitis virus type-2 infection in mice: an experimental model system of acute meningitis and hepatitis," *Experimental and Molecular Pathology*, vol. 71, no. 1, pp. 1–12, 2001.

[48] J. K. Fazakerley and M. J. Buchmeier, "Pathogenesis of virus-induced demyelination," *Advances in Virus Research*, vol. 42, pp. 249–324, 1993.

[49] M. Tardieu, O. Boespflug, and T. Barbe, "Selective tropism of a neurotropic coronavirus for ependymal cells, neurons, and meningeal cells," *Journal of Virology*, vol. 60, no. 2, pp. 574–582, 1986.

[50] S. Cheley, R. Anderson, M. J. Cupples, E. C. M. Lee Chana, and V. L. Morris, "Intracellular murine hepatitis virus-specific RNAs contain common sequences," *Virology*, vol. 112, no. 2, pp. 596–604, 1981.

[51] H. Vennema, G.-J. Godeke, J. W. A. Rossen, et al., "Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes," *EMBO Journal*, vol. 15, no. 8, pp. 2020–2028, 1996.

[52] F. Fischer, D. Peng, S. T. Hingley, S. R. Weiss, and P. S. Masters, "The internal open reading frame within the nucleocapsid gene of mouse hepatitis virus encodes a structural protein that is not essential for viral replication," *Journal of Virology*, vol. 71, no. 2, pp. 996–1003, 1997.

[53] D. A. Brian and R. S. Baric, "Coronavirus genome structure and replication," *Current Topics in Microbiology and Immunology*, vol. 287, pp. 1–30, 2005.

[54] A. E. Gorbalenya, E. V. Koonin, and M. M.-C. Lai, "Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi, $\alpha$- and coronaviruses," *FEBS Letters*, vol. 288, no. 1-2, pp. 201–205, 1991.

[55] W. Spaan, D. Cavanagh, and M. C. Horzinek, "Coronaviruses: structure and genome expression," *Journal of General Virology*, vol. 69, no. 12, pp. 2939–2952, 1988.

[56] G. S. Dveksler, C. W. Dieffenbach, C. B. Cardellichio, et al., "Several members of the mouse carcinoembryonic antigen-related glycoprotein family are functional receptors for the coronavirus mouse hepatitis virus-A59," *Journal of Virology*, vol. 67, no. 1, pp. 1–8, 1993.

[57] E. Prentice and M. R. Denison, "The cell biology of coronavirus infection," *Advances in Experimental Medicine and Biology*, vol. 494, pp. 609–614, 2001.

[58] K. L. Tyler, D. A. McPhee, and B. N. Fields, "Distinct pathways of viral spread in the host determined by reovirus S1 gene segment," *Science*, vol. 233, no. 4765, pp. 770–774, 1986.

[59] E. V. Agapov, I. Frolov, B. D. Lindenbach, B. M. Pragai, S. Schlesinger, and C. M. Rice, "Noncytopathic sindbis virus RNA vectors for heterologous gene expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 22, pp. 12989–12994, 1998.

[60] J.-C. Boyer and A.-L. Haenni, "Infectious transcripts and cDNA clones of RNA viruses," *Virology*, vol. 198, no. 1, pp. 415–426, 1994.

[61] S. Makino, J. G. Keck, S. A. Stohlman, and M. M. C. Lai, "High-frequency RNA recombination of murine coronaviruses," *Journal of Virology*, vol. 57, no. 3, pp. 729–737, 1986.

[62] C. A. Koetzner, M. M. Parker, C. S. Ricard, L. S. Sturman, and P. S. Masters, "Repair and mutagenesis of the genome of a deletion mutant of the coronavirus mouse hepatitis virus by targeted RNA recombination," *Journal of Virology*, vol. 66, no. 4, pp. 1841–1848, 1992.

[63] L. Kuo, G.-J. Godeke, M. J. B. Raamsman, P. S. Masters, and P. J. M. Rottier, "Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier," *Journal of Virology*, vol. 74, no. 3, pp. 1393–1406, 2000.

[64] P. S. Masters, "Reverse genetics of the largest RNA viruses," *Advances in Virus Research*, vol. 53, pp. 245–264, 1999.

[65] B. Yount, M. R. Denison, S. R. Weiss, and R. S. Baric, "Systematic assembly of a full-length infectious cDNA of mouse hepatitis virus strain A59," *Journal of Virology*, vol. 76, no. 21, pp. 11065–11078, 2002.

[66] E. F. Donaldson, B. Yount, A. C. Sims, S. Burkett, R. J. Pickles, and R. S. Baric, "Systematic assembly of a full-length infectious clone of human coronavirus NL63," *Journal of Virology*, vol. 82, no. 23, pp. 11948–11957, 2008.

[67] J. M. González, Z. Pénzes, F. Almazán, E. Calvo, and L. Enjuanes, "Stabilization of a full-length infectious cDNA clone of transmissible gastroenteritis coronavirus by insertion of an intron," *Journal of Virology*, vol. 76, no. 9, pp. 4655–4661, 2002.

[68] B. Yount, K. M. Curtis, E. A. Fritz, et al., "Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 22, pp. 12995–13000, 2003.

[69] R. S. Baric and A. C. Sims, "Development of mouse hepatitis virus and SARS-CoV infectious cDNA constructs," *Current Topics in Microbiology and Immunology*, vol. 287, pp. 229–252, 2005.

[70] E. Lavi, P. S. Fishman, M. K. Highkin, and S. R. Weiss, "Limbic encephalitis after inhalation of a murine coronavirus," *Laboratory Investigation*, vol. 58, no. 1, pp. 31–36, 1988.

[71] M. Tardieu, A. Goffinet, G. Harmant-van Rijckevorsel, and G. Lyon, "Ependymitis, leukoencephalitis, hydrocephalus, and thrombotic vasculitis following chronic infection by mouse hepatitis virus 3 (MHV 3)," *Acta Neuropathologica*, vol. 58, no. 3, pp. 168–176, 1982.

[72] A. A. Dandekar, G. F. Wu, L. Pewe, and S. Perlman, "Axonal damage is T cell mediated and occurs concomitantly with demyelination in mice infected with a neurotropic coronavirus," *Journal of Virology*, vol. 75, no. 13, pp. 6115–6120, 2001.

[73] J. Das Sarma, L. C. Kenyon, S. T. Hingley, and K. S. Shindler, "Mechanisms of primary axonal damage in a viral model of multiple sclerosis," *Journal of Neuroscience*, vol. 29, no. 33, pp. 10272–10280, 2009.

[74] M. A. Sussman, R. A. Shubin, S. Kyuwa, and S. A. Stohlman, "T-cell mediated clearance of mouse hepatitis virus strain JHM from the central nervous system," *Journal of Virology*, vol. 63, no. 7, pp. 3051–3056, 1989.

[75] S. A. Stohlman, C. C. Bergmann, R. C. Van der Veen, and D. R. Hinton, "Mouse hepatitis virus-specific cytotoxic T lymphocytes protect from lethal infection without eliminating virus from the central nervous system," *Journal of Virology*, vol. 69, no. 2, pp. 684–694, 1995.

[76] S. A. Stohlman, C. C. Bergmann, M. T. Lin, D. J. Cua, and D. R. Hinton, "CTL effector function within the central nervous system requires $CD4^+$ T cells," *Journal of Immunology*, vol. 160, no. 6, pp. 2896–2904, 1998.

[77] J. O. Fleming, F.-I. Wang, M. D. Trousdale, D. R. Hinton, and S. A. Stohlman, "Interaction of immune and central nervous systems: contribution of anti- viral Thy-1+ cells to demyelination induced by coronavirus JHM," *Regional Immunology*, vol. 5, no. 1, pp. 37–43, 1993.

[78] J. J. Houtman and J. O. Fleming, "Dissociation of demyelination and viral clearance in congenitally immunodeficient mice infected with murine coronavirus JHM," *Journal of NeuroVirology*, vol. 2, no. 2, pp. 101–110, 1996.

[79] A. A. Dandekar and S. Perlman, "Virus-induced demyelination in nude mice is mediated by $\gamma\delta$ T cells," *American Journal of Pathology*, vol. 161, no. 4, pp. 1255–1263, 2002.

[80] R. L. Knobler, P. W. Lampert, and M. B. A. Oldstone, "Virus persistence and recurring demyelination produced by a temperature-sensitive mutant of MHV-4," *Nature*, vol. 298, no. 5871, pp. 279–280, 1982.

[81] J. Das Sarma, L. Fu, J. C. Tsai, S. R. Weiss, and E. Lavi, "Demyelination determinants map to the spike glycoprotein gene of coronavirus mouse hepatitis virus," *Journal of Virology*, vol. 74, no. 19, pp. 9206–9213, 2000.

[82] J. Das Sarma, L. Fu, S. T. Hingley, M. M. C. Lai, and E. Lavi, "Sequence analysis of the S gene of recombinant MHV-2/A59 coronaviruses reveals three candidate mutations associated with demyelination and hepatitis," *Journal of NeuroVirology*, vol. 7, no. 5, pp. 432–436, 2001.

[83] M. V. Haspel, P. W. Lampert, and M. B. A. Oldstone, "Temperature-sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 75, no. 8, pp. 4033–4036, 1978.

[84] R. L. Knobler, M. V. Haspel, and M. B. A. Oldstone, "Mouse hepatitis virus type 4 (JHM strain)-induced fatal central nervous system disease. I. Genetic control and the murine neuron as the susceptible site of disease," *Journal of Experimental Medicine*, vol. 153, no. 4, pp. 832–843, 1981.

[85] Y. Li, L. Fu, D. M. Gonzales, and E. Lavi, "Coronavirus neurovirulence correlates with the ability of the virus to induce proinflammatory cytokine signals from astrocytes and microglia," *Journal of Virology*, vol. 78, no. 7, pp. 3398–3406, 2004.

[86] B. D. Pearce, M. V. Hobbs, T. S. McGraw, and M. J. Buchmeier, "Cytokine induction during T-cell-mediated clearance of mouse hepatitis virus from neurons in vivo," *Journal of Virology*, vol. 68, no. 9, pp. 5483–5495, 1994.

[87] B. Parra, D. R. Hinton, M. T. Lin, D. J. Cua, and S. A. Stohlman, "Kinetics of cytokine mRNA expression in the central nervous system following lethal and nonlethal coronavirus-induced acute encephalomyelitis," *Virology*, vol. 233, no. 2, pp. 260–270, 1997.

[88] M. J. Buchmeier and T. E. Lane, "Viral-induced neurodegenerative disease," *Current Opinion in Microbiology*, vol. 2, no. 4, pp. 398–402, 1999.

[89] N. Sun, D. Grzybicki, R. F. Castro, S. Murphy, and S. Perlman, "Activation of astrocytes in the spinal cord of mice chronically infected with a neutropic coronavirus," *Virology*, vol. 213, no. 2, pp. 482–493, 1995.

[90] J. G. Keck, L. H. Soe, S. Makino, S. A. Stohlman, and M. M. C. Lai, "RNA recombination of murine coronaviruses: recombination between fusion-positive mouse hepatitis virus A59 and fusion-negative mouse hepatitis virus 2," *Journal of Virology*, vol. 62, no. 6, pp. 1989–1998, 1988.

[91] R. L. Knobler, M. Dubois-Dalcq, M. V. Haspel, A. P. Claysmith, P. W. Lampert, and M. B. Oldstone, "Selective localization of wild type and mutant mouse hepatitis virus (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy," *Journal of Neuroimmunology*, vol. 1, p. 81, 1981.

[92] L. P. Weiner, R. T. Johnson, and R. M. Herndon, "Viral infections and demyelinating diseases," *The New England Journal of Medicine*, vol. 288, no. 21, pp. 1103–1110, 1973.

[93] Y. K. Yamada, K. Takimoto, M. Yabe, and F. Taguchi, "Acquired fusion activity of a murine coronavirus MHV-2 variant with mutations in the proteolytic cleavage site and the signal sequence of the S protein," *Virology*, vol. 227, no. 1, pp. 215–219, 1997.

[94] K. S. Shindler, L. C. Kenyon, M. Dutt, S. T. Hingley, and J. Das Sarma, "Experimental optic neuritis induced by a demyelinating strain of mouse hepatitis virus," *Journal of Virology*, vol. 82, no. 17, pp. 8882–8886, 2008.

[95] B. Kornek, M. K. Storch, R. Weissert, et al., "Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions," *American Journal of Pathology*, vol. 157, no. 1, pp. 267–276, 2000.

[96] K. S. Shindler, Y. Guan, E. Ventura, J. Bennett, and A. Rostami, "Retinal ganglion cell loss induced by acute optic neuritis in a relapsing model of multiple sclerosis," *Multiple Sclerosis*, vol. 12, no. 5, pp. 526–532, 2006.

[97] H. Shao, Z. Huang, S. L. Sun, H. J. Kaplan, and D. Sun, "Myelin/oligodendrocyte glycoprotein-specific T-cells induce severe optic neuritis in the C57B1/6 mouse," *Investigative Ophthalmology and Visual Science*, vol. 45, no. 11, pp. 4060–4065, 2004.

[98] K. S. Shindler, E. Ventura, T. S. Rex, P. Elliott, and A. Rostami, "SIRT1 activation confers neuroprotection in experimental optic neuritis," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 8, pp. 3602–3609, 2007.

[99] K. S. Shindler, E. Ventura, M. Dutt, and A. Rostami, "Inflammatory demyelination induces axonal injury and retinal ganglion cell apoptosis in experimental optic neuritis," *Experimental Eye Research*, vol. 87, no. 3, pp. 208–213, 2008.

[100] J. H. Noseworthy, C. Lucchinetti, M. Rodriguez, and B. G. Weinshenker, "Multiple sclerosis," *The New England Journal of Medicine*, vol. 343, no. 13, pp. 938–952, 2000.

[101] M. K. Guyton, E. A. Sribnick, S. K. Ray, and N. L. Banik, "A role for calpain in optic neuritis," *Annals of the New York Academy of Sciences*, vol. 1053, pp. 48–54, 2005.

[102] X. Qi, A. S. Lewin, L. Sun, W. W. Hauswirth, and J. Guy, "Suppression of mitochondrial oxidative stress provides

long-term neuroprotection in experimental optic neuritis," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 2, pp. 681–691, 2007.

[103] M. B. Sättler, D. Merkler, K. Maier, et al., "Neuroprotective effects and intracellular signaling pathways of erythropoietin in a rat model of multiple sclerosis," *Cell Death and Differentiation*, vol. 11, supplement 2, pp. S181–S192, 2004.

[104] M. Hobom, M. K. Storch, R. Weissert, et al., "Mechanisms and time course of neuronal degeneration in experimental autoimmune encephalomyelitis," *Brain Pathology*, vol. 14, no. 2, pp. 148–157, 2004.

[105] T. M. Gallagher and M. J. Buchmeier, "Coronavirus spike proteins in viral entry and pathogenesis," *Virology*, vol. 279, no. 2, pp. 371–374, 2001.

[106] S. T. Hingley, J. L. Gombold, E. Lavi, and S. R. Weiss, "MHV-A59 fusion mutants are attenuated and display altered hepatotropism," *Virology*, vol. 200, no. 1, pp. 1–10, 1994.

[107] J. J. Phillips, M. M. Chua, E. Lavi, and S. R. Weiss, "Pathogenesis of chimeric MHV4/MHV-A59 recombinant viruses: the murine coronavirus spike protein is a major determinant of neurovirulence," *Journal of Virology*, vol. 73, no. 9, pp. 7752–7760, 1999.

[108] J. J. Phillips, M. M. Chua, G. F. Rall, and S. R. Weiss, "Murine coronavirus spike glycoprotein mediates degree of viral spread, inflammation, and virus-induced immunopathology in the central nervous system," *Virology*, vol. 301, no. 1, pp. 109–120, 2002.

[109] J. J. Phillips and S. R. Weiss, "MHV neuropathogenesis: the study of chimeric S genes and mutations in the hypervariable region," *Advances in Experimental Medicine and Biology*, vol. 494, pp. 115–119, 2001.

[110] S. Navas, S.-H. Seo, M. M. Chua, et al., "Murine coronavirus spike protein determines the ability of the virus to replicate in the liver and cause hepatitis," *Journal of Virology*, vol. 75, no. 5, pp. 2452–2457, 2001.

[111] I. Leparc-Goffart, S. T. Hingley, M. M. Chua, J. Phillips, E. Lavi, and S. R. Weiss, "Targeted recombination within the spike gene of murine coronavirus mouse hepatitis virus-A59: Q159 is a determinant of hepatotropism," *Journal of Virology*, vol. 72, no. 12, pp. 9628–9636, 1998.

[112] J. C. Tsai, L. De Groot, J. D. Pinon, et al., "Amino acid substitutions within the heptad repeat domain 1 of murine coronavirus spike protein restrict viral antigen spread in the central nervous system," *Virology*, vol. 312, no. 2, pp. 369–380, 2003.

[113] F. Fischer, C. F. Stegen, C. A. Koetzner, and P. S. Masters, "Analysis of a recombinant mouse hepatitis virus expressing a foreign gene reveals a novel aspect of coronavirus transcription," *Journal of Virology*, vol. 71, no. 7, pp. 5148–5160, 1997.

[114] J. Das Sarma, E. Scheen, S.-H. Seo, M. Koval, and S. R. Weiss, "Enhanced green fluorescent protein expression may be used to monitor murine coronavirus spread in vitro and in the mouse central nervous system," *Journal of NeuroVirology*, vol. 8, no. 5, pp. 381–391, 2002.

[115] E. Ontiveros, L. Kuo, P. S. Masters, and S. Perlman, "Inactivation of expression of gene 4 of mouse hepatitis virus strain JHM does not affect virulence in the murine CNS," *Virology*, vol. 289, no. 2, pp. 230–238, 2001.

[116] J. Das Sarma, K. Iacono, L. Gard, et al., "Demyelinating and nondemyelinating strains of mouse hepatitis virus differ in their neural cell tropism," *Journal of Virology*, vol. 82, no. 11, pp. 5519–5526, 2008.

[117] P. De Camilli, P. E. Miller, F. Navone, W. E. Theurkauf, and R. B. Vallee, "Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence," *Neuroscience*, vol. 11, no. 4, pp. 819–846, 1984.

[118] A. Gadea, S. Schinelli, and V. Gallo, "Endothelin-1 regulates astrocyte proliferation and reactive gliosis via a JNK/c-Jun signaling pathway," *Journal of Neuroscience*, vol. 28, no. 10, pp. 2394–2408, 2008.

[119] J. M. M. Pasick, K. Kalicharran, and S. Dales, "Distribution and trafficking of JHM coronavirus structural proteins and virions in primary neurons and the OBL-21 neuronal cell line," *Journal of Virology*, vol. 68, no. 5, pp. 2915–2928, 1994.

[120] H. L. Weiner, M. L. Powers, and B. N. Fields, "Absolute linkage of virulence and central nervous system cell tropism of reoviruses to viral hemagglutinin," *Journal of Infectious Diseases*, vol. 141, no. 5, pp. 609–616, 1980.

[121] L. J. Zoecklein, K. D. Pavelko, J. Gamez, et al., "Direct comparison of demyelinating disease induced by the Daniel's strain and BeAn strain of Theiler's murine encephalomyelitis virus," *Brain Pathology*, vol. 13, no. 3, pp. 291–308, 2003.

[122] D. Moskophidis, M. Battegay, M. Van den Broek, E. Laine, U. Hoffmann-Rohrer, and R. M. Zinkernagel, "Role of virus and host variables in virus persistence or immunopathological disease caused by a non-cytolytic virus," *Journal of General Virology*, vol. 76, no. 2, pp. 381–391, 1995.

[123] D. Wodarz and D. C. Krakauer, "Defining CTL-induced pathology: implications for HIV," *Virology*, vol. 274, no. 1, pp. 94–104, 2000.

[124] K. C. MacNamara, M. M. Chua, P. T. Nelson, H. Shen, and S. R. Weiss, "Increased epitope-specific $CD8^+$ T cells prevent murine coronavirus spread to the spinal cord and subsequent demyelination," *Journal of Virology*, vol. 79, no. 6, pp. 3370–3381, 2005.

[125] L. W. Enquist, P. J. Husak, B. W. Banfield, and G. A. Smith, "Infection and spread of alphaherpesviruses in the nervous system," *Advances in Virus Research*, vol. 51, pp. 237–347, 1998.

[126] L. W. Enquist, M. J. Tomishima, S. Gross, and G. A. Smith, "Directional spread of an $\alpha$-herpesvirus in the nervous system," *Veterinary Microbiology*, vol. 86, no. 1-2, pp. 5–16, 2002.

[127] J.-P. Roussarie, C. Ruffie, J. M. Edgar, I. Griffiths, and M. Brahic, "Axon myelin transfer of a non-enveloped virus," *PLoS ONE*, vol. 2, no. 12, article e1331, 2007.

[128] E. Lavi, A. Suzumura, M. Hirayama, et al., "Coronavirus mouse hepatitis virus (MHV)-A59 causes a persistent, productive infection in primary glial cell cultures," *Microbial Pathogenesis*, vol. 3, no. 2, pp. 79–86, 1987.

[129] K. Kalicharran and S. Dales, "The murine coronavirus as a model of trafficking and assembly of viral proteins in neural tissue," *Trends in Microbiology*, vol. 4, no. 7, pp. 264–269, 1996.

[130] K. Kalicharran, D. Mohandas, G. Wilson, and S. Dales, "Regulation of the initiation of coronavirus JHM infection in primary oligodendrocytes and L-2 fibroblasts," *Virology*, vol. 225, no. 1, pp. 33–43, 1996.

[131] E. O. Major, K. Amemiya, C. S. Tornatore, S. A. Houff, and J. R. Berger, "Pathogenesis and molecular biology of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain," *Clinical Microbiology Reviews*, vol. 5, no. 1, pp. 49–73, 1992.

[132] S. M. Richardson-Burns, B. K. Kleinschmidt-DeMasters, R. L. DeBiasi, and K. L. Tyler, "Progressive multifocal leukoencephalopathy and apoptosis of infected oligodendrocytes in the central nervous system of patients with and without AIDS," *Archives of Neurology*, vol. 59, no. 12, pp. 1930–1936, 2002.

[133] D. S. Chen, M. Asanaka, F. S. Chen, J. E. Shively, and M. M. C. Lai, "Human carcinoembryonic antigen and biliary glycoprotein can serve as mouse hepatitis virus receptors," *Journal of Virology*, vol. 71, no. 2, pp. 1688–1691, 1997.

[134] K. Tan, B. D. Zelus, R. Meijers, et al., "Crystal structure of murine sCEACAM1a[1,4]: a coronavirus receptor in the CEA family," *EMBO Journal*, vol. 21, no. 9, pp. 2076–2086, 2002.

[135] T. M. Gallagher, M. J. Buchmeier, and S. Perlman, "Cell receptor-independent infection by a neurotropic murine coronavirus," *Virology*, vol. 191, no. 1, pp. 517–522, 1992.

[136] M. F. Frana, J. N. Behnke, L. S. Sturman, and K. V. Holmes, "Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion," *Journal of Virology*, vol. 56, no. 3, pp. 912–920, 1985.

[137] H. Kubo, Y. K. Yamada, and F. Taguchi, "Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus spike protein," *Journal of Virology*, vol. 68, no. 9, pp. 5403–5410, 1994.

[138] T. M. Gallagher and M. J. Buchmeier, "Coronavirus spike proteins in viral entry and pathogenesis," *Virology*, vol. 279, no. 2, pp. 371–374, 2001.

[139] S. Matsuyama and F. Taguchi, "Receptor-induced conformational changes of murine coronavirus spike protein," *Journal of Virology*, vol. 76, no. 23, pp. 11819–11826, 2002.

[140] B. D. Zelus, J. H. Schickli, D. M. Blau, S. R. Weiss, and K. V. Holmes, "Conformational changes in the spike glycoprotein of murine coronavirus are induced at 37°C either by soluble murine CEACAM1 receptors or by pH8," *Journal of Virology*, vol. 77, no. 2, pp. 830–840, 2003.

[141] Z. Luo and S. R. Weiss, "Roles in cell-to-cell fusion of two conserved hydrophobic regions in the murine coronavirus spike protein," *Virology*, vol. 244, no. 2, pp. 483–494, 1998.

[142] C. A. M. de Haan, P. S. Masters, X. Shen, S. Weiss, and P. J. M. Rottier, "The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host," *Virology*, vol. 296, no. 1, pp. 177–189, 2002.

[143] B. Charley and H. Laude, "Induction of alpha interferon by transmissible gastroenteritis coronavirus: role of transmembrane glycoprotein E1," *Journal of Virology*, vol. 62, no. 1, pp. 8–11, 1988.

[144] C. A. M. de Haan, M. de Wit, L. Kuo, et al., "O-glycosylation of the mouse hepatitis coronavirus membrane protein," *Virus Research*, vol. 82, no. 1-2, pp. 77–81, 2002.

[145] A. Hirano, A. H. Wang, A. F. Gombart, and T. C. Wong, "The matrix proteins of neurovirulent subacute sclerosing panencephalitis virus and its acute measles virus progenitor are functionally different," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 18, pp. 8745–8749, 1992.

[146] Q. Ning, M. Liu, P. Kongkham, et al., "The nucleocapsid protein of murine hepatitis virus type 3 induces transcription of the novel fgl2 prothrombinase gene," *Journal of Biological Chemistry*, vol. 274, no. 15, pp. 9930–9936, 1999.

[147] P. S. Masters, C. A. Koetzner, C. A. Kerr, and Y. Heo, "Optimization of targeted RNA recombination and mapping of a novel nucleocapsid gene mutation in the coronavirus mouse hepatitis virus," *Journal of Virology*, vol. 68, no. 1, pp. 328–337, 1994.

[148] S. R. Weiss, P. W. Zoltick, and J. L. Leibowitz, "The ns 4 gene of mouse hepatitis virus (MHV), strain A 59 contains two ORFs and thus differs from ns 4 of the JHM and S strains," *Archives of Virology*, vol. 129, no. 1–4, pp. 301–309, 1993.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 478461, 8 pages
doi:10.1155/2010/478461

*Review Article*

# Immunogenetics and the Pathological Mechanisms of Human T-Cell Leukemia Virus Type 1- (HTLV-1-)Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

**Mineki Saito**

*Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan*

Correspondence should be addressed to Mineki Saito, mineki@med.u-ryukyu.ac.jp

Received 8 October 2009; Accepted 4 January 2010

Academic Editor: Marylou V. Solbrig



Human T-cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease only in a minority of infected individuals: the malignancy known as adult T-cell leukemia (ATL) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, accumulating evidence suggests that complex virus-host interactions play an important role in determining the risk of HAM/TSP. This review focuses on the role of the immune response in controlling or limiting viral persistence in HAM/TSP patients and the reason why some HTLV-1-infected people develop HAM/TSP whereas the majority remains asymptomatic carriers of the virus.

## 1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection is of particular interest to the field of immunology as well as microbiology because HTLV-1 is never eliminated from the host in spite of vigorous cellular and humoral immune responses against the virus but causes no disease in vast majority of infected subjects (asymptomatic carriers:ACs). Although only approximately 2%-3% develop adult T cell leukemia (ATL) [1, 2] and another 0.25%–3.8% develop chronic inflammatory diseases involving the central nervous system (HTLV-1-associated myelopathy/tropical spastic paraparesis: HAM/TSP) [3, 4], evaluation of the individual risk for developing diseases in each ACs would certainly be of considerable importance especially in HTLV-1 endemic area such as southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [5]. However, many fundamental questions are remained to be solved. First, how does HTLV-1 persist in the individual host in spite of strong host immune response? Second, why do some HTLV-1-infected people develop consequent diseases such as ATL or HAM/TSP, whereas the majority remains asymptomatic carriers of the virus? Third, how is the inflammatory lesion in HAM/TSP initiated and maintained, and why is the inflammation specifically in thoracic spinal cord? This review summarizes the past and recent works for HAM/TSP attempting to resolve each of these questions.

## 2. Clinical and Pathological Features of HAM/TSP

HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae* and infects 10–20 million people worldwide [6–8]. HTLV-1 can be transmitted through sexual contact [9], injection drug use [8], and breastfeeding from mother to child [10, 11]. Although HTLV-1 infection is associated with a range of nonmalignant chronic inflammatory diseases in the eyes, the lungs, or the skeletal muscles [7], HAM/TSP is the bestrecognized with chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [12]. To date, more

than 3,000 cases of HAM/TSP patients have been reported in HTLV-1 endemic areas. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1 endemic area. Among ACs, the lifetime risk of developing HAM/TSP, which is different among different ethnic groups, ranges between 0.25% and 4%. It has been reported that the annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times higher risk for women in both populations [13–16].

Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level [17–19]. Loss of myelin and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis. The presence of atypical lymphocytes (so-called "flower cells") in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal bands, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-6 and interferon (IFN)-$\gamma$, and an increased intrathecal antibody synthesis specific for HTLV-1 antigens have also been described [20]. Clinical progression of HAM/TSP is associated with increased proviral load in individual patients, and the ratio of proviral loads in CSF cells/in peripheral blood mononuclear cells (PBMCs) is significantly associated with clinically progressive disease [21]. The major histocompatibility complex (MHC) class I tetramer analysis of lymphocytes isolated from the CSF of HAM/TSP patients showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted $CD8^+$ lymphocytes compared to those of PBMCs [22]. Therefore, an increased proliferation or migration of HTLV-1-infected and/or HTLV-1-specific lymphocytes to the central nervous system (CNS) might be closely associated with HAM/TSP pathogenesis [23].

## 3. Risk Factors for HAM/TSP

*3.1. Host Genetics.* Previous population association study of 202 cases of HAM/TSP and 243 ACs in Kagoshima, HTLV-1 endemic southern Japan, revealed that one of the major risk factors is the HTLV-1 proviral load. The median proviral load was more than ten times higher in HAM/TSP patients than in ACs, and a high proviral load was also associated with an increased risk of progression to disease [24]. Higher proviral load in HAM/TSP patients than in ACs was observed in other endemic area, such as the Caribbean [25], South America [26], and the Middle East [27]. It was suggested that genetic factors such as human leukocyte antigen (HLA) are related to the high proviral load in HAM/TSP patients and genetic relatives. In southern Japan, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 proviral load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposes to HAM/TSP in the same population [28, 29]. Since the function of class I HLA proteins is to present antigenic peptides to cytotoxic T lymphocytes (CTL), these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of HTLV-1 proviral load and the risk of HAM/TSP. Further analysis to look at nonHLA host genetic factors revealed that nonHLA gene polymorphism also affects the risk for developing HAM/TSP. For example, the TNF-$\alpha$ promoter $-863$ A allele [30], and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter [31] predisposed to HAM/TSP, whereas IL-10 -592 A [32], Stromal derived factor (SDF)-1 +801A [30] and IL-15 +191 C alleles [30] conferred protection against HAM/TSP. The polymorphisms of MMP-9 and IL-10 promoter each linked to the HTLV-1-encoded transactivator Tax-mediated transcriptional activity of each gene [31, 32].

*3.2. HTLV-1 Genotype.* Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP [33]. The *tax* subgroup A that belongs to cosmopolitan subtype A was more frequently observed in HAM/TSP patients and this effect was independent of protective allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B that belongs to cosmopolitan subtype B but not against *tax* subgroup A in Japanese population [33]. Interestingly, HLA-A*02 appears not to give protection against infection with cosmopolitan subtype A in a population in Iran [27]. These findings suggest that both host genetic factors and HTLV-1 subgroup play a part in determining the risk of HAM/TSP.

## 4. The Immune Response to HTLV-1

*4.1. The Humoral Immune Response.* In HTLV-1 infection, anti-HTLV-1 antibody that often includes IgM is detected in all infected individuals, either ACs or patients with HAM/TSP [34]. It has been reported that HAM/TSP patients generally had higher anti-HTLV-1 antibody titer than ACs with the similar HTLV-1 proviral load [34–36]. These data suggest that there was persistent expression of HTLV-1 proteins in vivo and the existence of an augmented humoral immune response to HTLV-1 in HAM/TSP patients. Interestingly, although antibody responses to the immunodominant epitopes of the HTLV-1 Envelope (Env) proteins were similar in all of three clinical groups (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was highest in HAM/TSP (71%–93%) than ATL patients (4%–31%) or ACs (27%–37%) [37]. In 2002, Levin et al. reported that antibodies that recognize HTLV-1 Tax protein can cross-react with a heterogenous nuclear riboprotein (hnRNP)-A1, suggesting intriguing evidence for antigen mimicry in HTLV-1 infection [38]. However, since the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed [39] and is not normally accessible to antibody

attack, it is unlikely that anti-Tax antibody explains the onset or initial tissue damage of HAM/TSP. Rather, anti-Tax antibody might be associated with subsequent inflammation following initial tissue damage and disruption of blood brain barrier, which is probably caused by the antiviral immune responses to HTLV-1 and induces the release of autoantigens.

*4.2. The Natural Killer (NK) Cell Response.* Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^+CD16^+$ subset) than ACs, although the results were not normalized with respect to the proviral load [40]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, the synthetic tetramers of HLA-E with the HLA-G signal sequence peptide were used to identify NK cells in HAM/TSP patients [41]. The results clearly showed a lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [40], this reduction in frequency was particularly notable in the $CD3^+$ cells whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and ACs [41]. Recent data also suggest that the frequency of invariant NKT cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [42, 43]. These findings indicated that the activity of the NK or NKT cell response was associated with the presence or absence of HAM/TSP. Interestingly, previous uncontrolled preliminary trial of viable *Lactobacillus casei* strain Shirota containing fermented milk for HAM/TSP patients resulted in significant increase of NK cell activity with improvements in clinical symptoms [44]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP.

*4.3. The Regulatory T Cells (Tregs).* It has been reported that HTLV-1 preferentially and persistently infects $CD4^+CD25^+$ lymphocytes in vivo [45], which contains the majority of the $Foxp3^+$ Tregs [46]. In HAM/TSP patients, the percentage of $Foxp3^+$ Tregs in $CD4^+CD25^+$ cells is lower than that in ACs and uninfected healthy controls [45, 47] whereas the percentage of $Foxp3^+$ cells in the $CD4^+$ population tended to be higher in the HAM/TSP patients than in the ACs [48–50]. As CD25 is induced by HTLV-1 Tax oncoprotein [51], it is most likely that the proportion of $Foxp3^+$ cells falls in the $CD4^+CD25^+$ population, which contain both Tregs and activated nonTregs, in HTLV-1-infected individuals especially HAM/TSP patients. Interestingly, the frequency of HTLV-1 negative $Foxp3^+CD4^+$ cells positively correlated with the HTLV-1 proviral load [23, 49] and the CTL activity negatively correlated with the frequency of HTLV-1 negative $Foxp3^+CD4^+$ cells [49]. These data suggest that an increase in HTLV-1 negative $FoxP3^+CD4^+$ Tregs is one of the chief determinants of the efficiency of T cell mediated immune control of HTLV-1. If such Tregs reduce CTL activity, which in turn increases the HTLV-1 proviral load, this activity increases the risk for developing HAM/TSP.

*4.4. The $CD4^+$ Helper T Cell Response.* It is well known that antiviral $CD4^+$ T cell responses are of central importance in driving B-cell and $CD8^+$ T-cell responses in vivo. The HTLV-1 antigen most commonly recognized by $CD4^+$ T cells is the Env protein [52, 53], in contrast with the immunodominance of Tax in the $CD8^+$ T cell response [54–56]. At a similar proviral load, patients with HAM/TSP had significantly increased frequency of virus-specific $CD4^+$ T-cells compared to that of ACs [53, 57]. The antiviral T helper (Th)1 phenotype is also dominant among HTLV-1-specific $CD4^+$ T cells in both ACs and patients with HAM/TSP [58], and there is a higher frequency of IFN-$\gamma$, TNF-$\alpha$, and IL-2 production by $CD4^+$ T cells in patients with HAM/TSP compared to ACs of a similar proviral load [58, 59]. A role for CD4+ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in southern Japan [28, 29] and northeastern Iran [27]. Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380–394 peptide was used to analyze Env-specific $CD4^+$ T cells directly ex vivo [57]. The results showed that the frequency of $tetramer^+CD4^+$ T cells was significantly higher in HAM/TSP patients than in ACs with similar proviral load. Moreover, direct ex vivo analysis of $tetramer^+CD4^+$ T cells from two unrelated DRB1*0101 positive HAM/TSP patients indicated that certain T cell receptor (TCR) V$\beta$s were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These data suggest that the observed increase in virus-specific $CD4^+$ T cells in HAM/TSP patients, which may contribute to $CD4^+$ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected $CD4^+$ T cells but was the result of in vivo selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 $tetramer^+$ $CD8^+$ T cells [60] and muscle infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [61].

*4.5. The Cytotoxic T Lymphocyte (CTL) Response.* Previous reports indicated that the HTLV-1-specific $CD8^+$ CTL is typically abundant, chronically activated, and mainly targeted to the viral transactivator protein Tax [62]. Also, as already mentioned, the median proviral load in PBMCs of HAM/TSP patients was more than ten times higher than that in ACs, and a high proviral load was also associated with an increased risk of progression to disease [24]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower proviral load and a lower risk of HAM/TSP [28, 29], and $CD8^+$ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals [63]. These data have raised the hypothesis that the class I-restricted $CD8^+$ CTL response

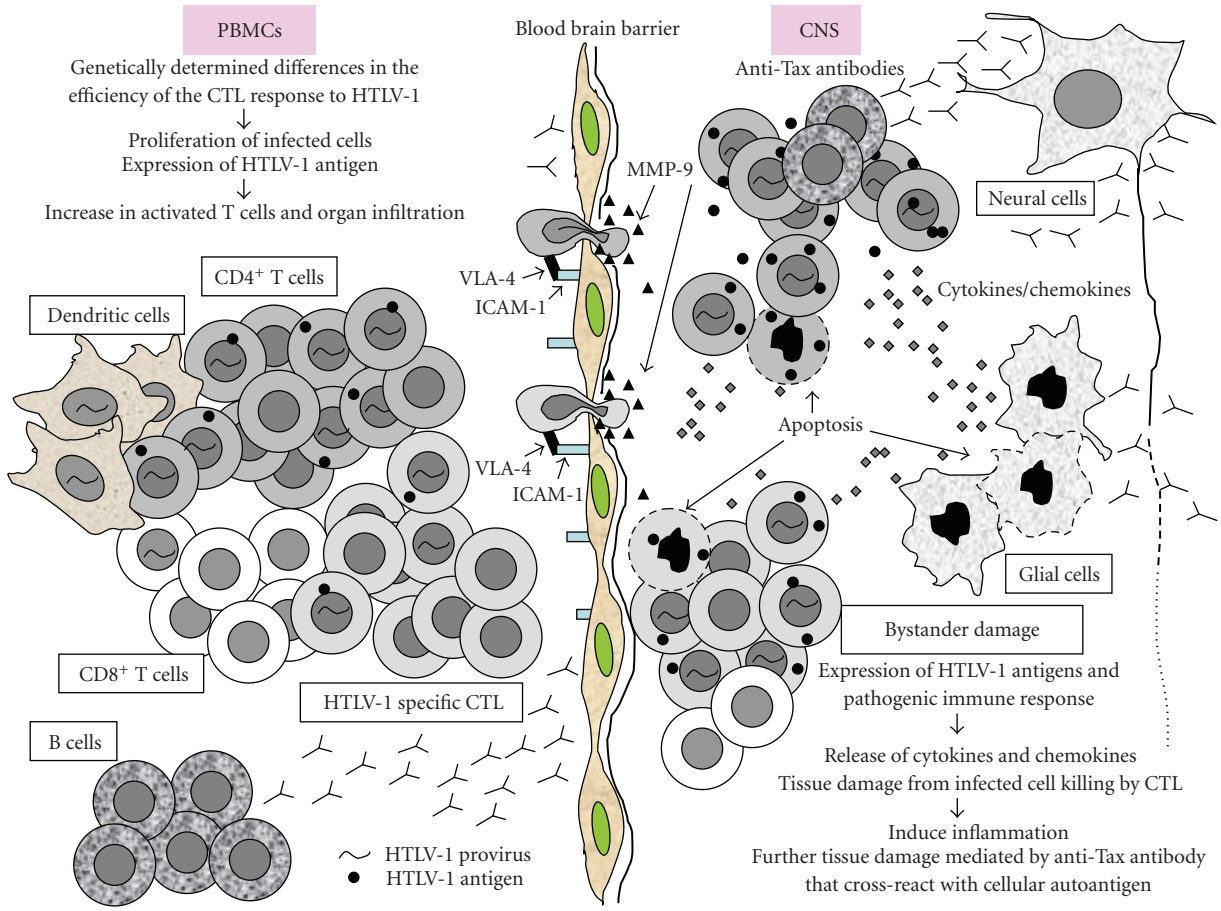



Figure 1: *Hypothesis for the pathogenesis of Human T-cell leukemia virus type 1- (HTLV-1-)associated myelopathy/tropical spastic paraparesis (HAM/TSP)*. In patients with HAM/TSP, genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression, leading in turn to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-reacts with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

plays a critical part in limiting HTLV-1 replication in vivo and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk for developing HAM/TSP. However, since the frequency of HTLV-1-specific $CD8^+$ T cells was significantly elevated in HAM/TSP patients than in ACs [64, 65] and these cells have the potential to produce proinflammatory cytokines [66], there is a debate on the role of HTLV-1-specific-$CD8^+$ T cells, that is, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP or whether the dominant effect of such cells in vivo is protective against disease, although these two mechanisms are not mutually exclusive. Recently, Sabouri et al. reported that a frequency of $CD8^+$ T cells that were negative for costimulatory molecules such as CD27, CD28, CD80, CD86, and CD152 was significantly higher in patients with HAM/TSP than in age-matched uninfected controls, but there was no such difference between ACs and uninfected controls [67]. They also found a significantly lower frequency of perforin$^+$ cells and granzyme $B^+$ cells in the $CD8^+$ T cells in HTLV-1-infected subjects than in uninfected controls, although there was no significant difference between patients with HAM/TSP and ACs. Furthermore, the lytic capacity of HTLV-1-specific CTL between HAM/TSP and ACs estimated by CD107a mobilization assay showed the significantly lower CD107a staining in HTLV-1-specific CTL in HAM/TSP than ACs. These findings suggest that patients with HAM/TSP have a high frequency of HTLV-1-specific $CD8^+$ T cells with poor lytic capacity, whereas ACs has a lower frequency of cells with high lytic capacity.

*4.6. Dendritic Cells (DCs).* Dendritic cells are antigen-presenting cells which play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from HAM/TSP patients were infected with HTLV-1 [68], and the development of HAM/TSP is associated with rapid maturation of DCs [69]. One of the hallmarks of HTLV-1 infection is the in vitro proliferation of PBMCs when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous

lymphocyte proliferation (SLP), and in HAM/TSP patients, the levels of SLP reflect the severity of the disease [70, 71]. Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolishes SPL while supplementing DCs, but not B cells nor macrophages restore proliferation [68]. DC dependent mechanism of SLP was further supported by data showing that antibodies to MHC class II, CD86, and CD58 can block SPL [72]. Recently, Jones et al. had demonstrated that human-derived both myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1, and HTLV-1-infected DCs can rapidly transfer virus to autologous primary $CD4^+$ T cells [73]. Furthermore, in contrast to the previous report that $CD4^+CD25^+$ T cells are responsible for the stimulation of Tax-specific $CD8^+$ T cells [74], it was recently demonstrated that, compared to the $CD4^+CD25^+$ T cells, the DCs are the major cell type responsible for the generation and maintenance of the Tax-specific $CD8^+$ T cells both in vitro and in vivo [75]. These findings suggest that the interaction of DCs with HTLV-1 is also crucial for the pathogenesis of HAM/TSP.

*4.7. The Other Reservoirs of HTLV-1.* Previous studies have indicated that only a small proportion of the monocyte-macrophage lineage cells are infected with HTLV-1 in peripheral blood [76] and that there has been no direct evidence indicating that HTLV-1-infected cells of the monocyte-macrophage lineage cells are present in the CNS [77]. However, monocyte-macrophage lineage cells may also play important roles in the pathogenesis of HAM/TSP, since it has been shown that the activation of macrophage and microglial cells within the CNS closely correlated with the proviral load within the CNS of HAM/TSP patients [78]. Meanwhile, it was also shown that a vast majority of bone marrow cells from HAM/TSP patients are positive for HTLV-1 proviral DNA but negative for viral RNA expression [79], whereas no HTLV-1 proviral DNA positive $CD34^+$ hematopoietic progenitor cells were detected in ATL patients [80]. These results suggest that HTLV-1-infected cells within the bone marrow may be a reservoir of HTLV-1 in HAM/TSP patients and play an important role in the etiology of neuroinflammation observed in HAM/TSP [77].

## 5. Conclusions

As shown in Figure 1, accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development.

## Acknowledgments

The author thanks the Ministry of Health, Labor and Welfare, Japan (Health Labour Sciences Research Grant) and the Japan Society for the Promotion of Science (JSPS) (Grant-in-Aid for Scientific Research, ref 21590512) for financial supports.

## References

[1] Y. Hinuma, Nagata, M. Hanaoka, et al., "Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 10, pp. 6476–6480, 1981.

[2] M. Yoshida, M. Seiki, K. Yamaguchi, and K. Takatsuki, "Monoclonal integration of human T-cell leukemia provirus in all primary tumors of adult T-cell leukemia suggests causative role of human T-cell leukemia virus in the disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 8, pp. 2534–2537, 1984.

[3] M. Osame, K. Usuku, S. Izumo, et al., "HTLV-I associated myelopathy, a new clinical entity," *The Lancet*, vol. 1, no. 8488, pp. 1031–1032, 1986.

[4] A. Gessain, F. Barin, J. C. Vernant, et al., "Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis," *The Lancet*, vol. 2, no. 8452, pp. 407–410, 1985.

[5] K. Verdonck, E. González, S. Van Dooren, A.-M. Vandamme, G. Vanham, and E. Gotuzzo, "Human T-lymphotropic virus 1: recent knowledge about an ancient infection," *The Lancet Infectious Diseases*, vol. 7, no. 4, pp. 266–281, 2007.

[6] G. de The and R. Bomford, "An HTLV-I vaccine: why, how, for whom?" *AIDS Research and Human Retroviruses*, vol. 9, no. 5, pp. 381–386, 1993.

[7] T. Uchiyama, "Human T cell leukemia virus type I (HTLV-I) and human diseases," *Annual Review of Immunology*, vol. 15, pp. 15–37, 1997.

[8] F. A. Proietti, A. B. F. Carneiro-Proietti, B. C. Catalan-Soares, and E. L. Murphy, "Global epidemiology of HTLV-I infection and associated diseases," *Oncogene*, vol. 24, no. 39, pp. 6058–6068, 2005.

[9] D. F. Roucoux, B. Wang, D. Smith, et al., "A prospective study of sexual transmission of human T lymphotropic virus (HTLV)-I and HTLV-II," *Journal of Infectious Diseases*, vol. 191, no. 9, pp. 1490–1497, 2005.

[10] S. Hino, K. Yamaguchi, S. Katamine, et al., "Mother-to-child transmission of human T-cell leukemia virus type-I," *Japanese Journal of Cancer Research*, vol. 76, no. 6, pp. 474–480, 1985.

[11] K. Kinoshita, T. Amagasaki, S. Hino, et al., "Milk-borne transmission of HTLV-I from carrier mothers to their children," *Japanese Journal of Cancer Research*, vol. 78, no. 7, pp. 674–680, 1987.

[12] M. Nakagawa, K. Nakahara, Y. Maruyama, et al., "Therapeutic trials in 200 patients with HTLV-I-associated myelopathy/tropical spastic paraparesis," *Journal of NeuroVirology*, vol. 2, no. 5, pp. 345–355, 1996.

[13] M. Osame, R. Janssen, H. Kubota, et al., "Nationwide survey of HTLV-I-asscociated myelopathy in Japan: association with blood transfusion," *Annals of Neurology*, vol. 28, no. 1, pp. 50–56, 1990.

[14] M. Hisada, S. O. Stuver, A. Okayama, et al., "Persistent paradox of natural history of human T lymphotropic virus type I: parallel analyses of Japanese and Jamaican carriers," *Journal of Infectious Diseases*, vol. 190, no. 9, pp. 1605–1609, 2004.

[15] M. Nakagawa, S. Izumo, S. Ijichi, et al., "HTLV-I-associated myelopathy: analysis of 213 patients based on clinical features

and laboratory findings," *Journal of Neurovirology*, vol. 1, no. 1, pp. 50–61, 1995.

[16] A. Kramer, E. M. Maloney, O. S. C. Morgan, et al., "Risk factors and cofactors for human T-cell lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in Jamaica," *American Journal of Epidemiology*, vol. 142, no. 11, pp. 1212–1220, 1995.

[17] Y. Iwasaki, "Pathology of chronic myelopathy associated with HTLV-I infection (HAM/TSP)," *Journal of the Neurological Sciences*, vol. 96, no. 1, pp. 103–123, 1990.

[18] A. Yoshioka, G. Hirose, Y. Ueda, Y. Nishimura, and K. Sakai, "Neuropathological studies of the spinal cord in early stage HTLV-I-associated myelopathy (HAM)," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 56, no. 9, pp. 1004–1007, 1993.

[19] S. Izumo, F. Umehara, and M. Osame, "HTLV-I-associated myelopathy," *Neuropathology*, vol. 20, supplement, pp. S65–S68, 2000.

[20] S. Jacobson, "Immunopathogenesis of human T cell lymphotropic virus type I-associated neurologic disease," *Journal of Infectious Diseases*, vol. 186, supplement 2, pp. S187–S192, 2002.

[21] N. Takenouchi, Y. Yamano, K. Usuku, M. Osame, and S. Izumo, "Usefulness of proviral load measurement for monitoring of disease activity in individual patients with human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis," *Journal of NeuroVirology*, vol. 9, no. 1, pp. 29–35, 2003.

[22] M. Nagai, Y. Yamano, M. B. Brennan, C. A. Mora, and S. Jacobson, "Increased HTLV-I proviral load and preferential expansion of HTLV-I Tax-specific $CD8^+$ T cells in cerebrospinal fluid from patients with HAM/TSP," *Annals of Neurology*, vol. 50, no. 6, pp. 807–812, 2001.

[23] D. Hayashi, R. Kubota, N. Takenouchi, et al., "Accumulation of human T-lymphotropic virus type I (HTLV-I)-infected cells in the cerebrospinal fluid during the exacerbation of HTLV-I-associated myelopathy," *Journal of NeuroVirology*, vol. 14, no. 5, pp. 459–463, 2008.

[24] M. Nagai, K. Usuku, W. Matsumoto, et al., "Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP," *Journal of NeuroVirology*, vol. 4, no. 6, pp. 586–593, 1998.

[25] A. Manns, W. J. Miley, R. J. Wilks, et al., "Quantitative proviral DNA and antibody levels in the natural history of HTLV-I infection," *Journal of Infectious Diseases*, vol. 180, no. 5, pp. 1487–1493, 1999.

[26] V. Adaui, K. Verdonck, I. Best, et al., "SYBR green-based quantitation of human T-lymphotropic virus type 1 proviral load in Peruvian patients with neurological disease and asymptomatic carriers: influence of clinical status, sex, and familial relatedness," *Journal of NeuroVirology*, vol. 12, no. 6, pp. 456–465, 2006.

[27] A. H. Sabouri, M. Saito, K. Usuku, et al., "Differences in viral and host genetic risk factors for development of human T-cell lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis between Iranian and Japanese HTLV-1-infected individuals," *Journal of General Virology*, vol. 86, no. 3, pp. 773–781, 2005.

[28] K. J. M. Jeffery, A. A. Siddiqui, M. Bunce, et al., "The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection," *Journal of Immunology*, vol. 165, no. 12, pp. 7278–7284, 2000.

[29] K. J. M. Jeffery, K. Usuku, S. E. Hall, et al., "HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3848–3853, 1999.

[30] A. M. Vine, A. D. Witkover, A. L. Lloyd, et al., "Polygenic control of human T lymphotropic virus type I (HTLV-I) provirus load and the risk of HTLV-I-associated myelopathy/tropical spastic paraparesis," *Journal of Infectious Diseases*, vol. 186, no. 7, pp. 932–939, 2002.

[31] D. Kodama, M. Saito, W. Matsumoto, et al., "Longer dinucleotide repeat polymorphism in matrix metalloproteinase-9 (MMP-9) gene promoter which correlates with higher HTLV-I Tax mediated transcriptional activity influences the risk of HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP)," *Journal of Neuroimmunology*, vol. 156, no. 1-2, pp. 188–194, 2004.

[32] A. H. Sabouri, M. Saito, A. L. Lloyd, et al., "Polymorphism in the interleukin-10 promoter affects both provirus load and the risk of human T lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis," *Journal of Infectious Diseases*, vol. 190, no. 7, pp. 1279–1285, 2004.

[33] Y. Furukawa, M. Yamashita, K. Usuku, S. Izumo, M. Nakagawa, and M. Osame, "Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the tax gene and their association with different risks for HTLV-I-associated myelopathy/tropical spastic paraparesis," *Journal of Infectious Diseases*, vol. 182, no. 5, pp. 1343–1349, 2000.

[34] J. Kira, M. Nakamura, T. Sawada, et al., "Antibody titers to HTLV-I-$p40^{tax}$ protein and gag-env hybrid protein in HTLV-I-associated myelopathy/tropical spastic paraparesis: correlation with increased HTLV-I proviral DNA load," *Journal of the Neurological Sciences*, vol. 107, no. 1, pp. 98–104, 1992.

[35] S. Ishihara, A. Okayama, S. Stuver, et al., "Association of HTLV-I antibody profile of asymptomatic carriers with proviral DNA levels of peripheral blood mononuclear cells," *Journal of Acquired Immune Deficiency Syndromes*, vol. 7, no. 2, pp. 199–203, 1994.

[36] K. Nagasato, T. Nakamura, S. Shirabe, et al., "Presence of serum anti-human T-lymphotropic virus type I (HTLV-I) IgM antibodies means persistent active replication of HTLV-I in HTLV-I-associated myelopathy," *Journal of the Neurological Sciences*, vol. 103, no. 2, pp. 203–208, 1991.

[37] R. B. Lal, C.-Z. Giam, J. E. Coligan, and D. L. Rudolph, "Differential immune responsiveness to the immunodominant epitopes of regulatory proteins (tax and rex) in human T cell lymphotropic virus type I-associated myelopathy," *Journal of Infectious Diseases*, vol. 169, no. 3, pp. 496–503, 1994.

[38] M. C. Levin, S. M. Lee, F. Kalume, et al., "Autoimmunity due to molecular mimicry as a cause of neurological disease," *Nature Medicine*, vol. 8, no. 5, pp. 509–513, 2002.

[39] G. Dreyfuss, M. J. Matunis, S. Pinol-Roma, and C. G. Burd, "hnRNP proteins and the biogenesis of mRNA," *Annual Review of Biochemistry*, vol. 62, pp. 289–321, 1993.

[40] F. Yu, Y. Itoyama, K. Fujihara, and I. Goto, "Natural killer (NK) cells in HTLV-I-associated myelopathy/tropical spastic paraparesis-decrease in NK cell subset populations and activity in HTLV-I seropositive individuals," *Journal of Neuroimmunology*, vol. 33, pp. 121–128, 1991.

[41] M. Saito, V. M. Braud, P. Goon, et al., "Low frequency of $CD94/NKG2A^+$ T lymphocytes in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis, but not in asymptomatic carriers," *Blood*, vol. 102, no. 2, pp. 577–584, 2003.

[42] K. Azakami, T. Sato, N. Araya, et al., "Severe loss of invariant NKT cells exhibiting anti-HTLV-1 activity in patients with HTLV-1-associated disorders," *Blood*, vol. 114, no. 15, pp. 3208–3215, 2009.

[43] L. C. Ndhlovu, J. E. Snyder-Cappione, K. I. Carvalho, et al., "Lower numbers of circulating natural killer T (NK T) cells in individuals with human T lymphotropic virus type 1 (HTLV-1) associated neurological disease," *Clinical and Experimental Immunology*, vol. 158, no. 3, pp. 294–299, 2009.

[44] T. Matsuzaki, M. Saito, K. Usuku, et al., "A prospective uncontrolled trial of fermented milk drink containing viable *Lactobacillus casei* strain Shirota in the treatment of HTLV-1 associated myelopathy/tropical spastic paraparesis," *Journal of the Neurological Sciences*, vol. 237, no. 1-2, pp. 75–81, 2005.

[45] Y. Yamano, N. Takenouchi, H.-C. Li, et al., "Virus-induced dysfunction of $CD4^+CD25^+$ T cells in patients with HTLV-I-associated neuroimmunological disease," *Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1361–1368, 2005.

[46] S. Sakaguchi, M. Ono, R. Setoguchi, et al., "$Foxp3^+CD25^+CD4^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease," *Immunological Reviews*, vol. 212, pp. 8–27, 2006.

[47] U. Oh, C. Grant, C. Griffith, K. Fugo, N. Takenouchi, and S. Jacobson, "Reduced Foxp3 protein expression is associated with inflammatory disease during human T lymphotropic virus type 1 infection," *Journal of Infectious Diseases*, vol. 193, no. 11, pp. 1557–1566, 2006.

[48] D. Hayashi, R. Kubota, N. Takenouchi, et al., "Reduced Foxp3 expression with increased cytomegalovirus-specific CTL in HTLV-I-associated myelopathy," *Journal of Neuroimmunology*, vol. 200, no. 1-2, pp. 115–124, 2008.

[49] F. Toulza, A. Heaps, Y. Tanaka, G. P. Taylor, and C. R. M. Bangham, "High frequency of $CD4^+FoxP3^+$ cells in HTLV-1 infection: inverse correlation with HTLV-l-specific CTL response," *Blood*, vol. 111, no. 10, pp. 5047–5053, 2008.

[50] I. Best, G. López, K. Verdonck, et al., "IFN-$\gamma$ production in response to Tax 161-233, and frequency of $CD4^+$ $Foxp3^+$ and Lin-HLA-DRhigh $CD123^+$ cells, discriminate HAM/TSP patients from asymptomatic HTLV-1-carriers in a Peruvian population," *Immunology*, vol. 128, no. 1, part 2, pp. e777–e786, 2009.

[51] J. Inoue, M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida, "Induction of interleukin 2 receptor gene expression by p40x encoded by human T-cell leukemia virus type 1," *EMBO Journal*, vol. 5, no. 11, pp. 2883–2888, 1986.

[52] B. Kitze, K. Usuku, Y. Yamano, et al., "Human $CD4^+$ T lymphocytes recognize a highly conserved epitope of human T lymphotropic virus type 1 (HTLV-1) env gp21 restricted by HLA DRB1*0101," *Clinical and Experimental Immunology*, vol. 111, no. 2, pp. 278–285, 1998.

[53] P. K. C. Goon, T. Igakura, E. Hanon, et al., "Human T cell lymphotropic virus type I (HTLV-I)-specific $CD4^+$ T cells: immunodominance hierarchy and preferential infection with HTLV-I," *Journal of Immunology*, vol. 172, no. 3, pp. 1735–1743, 2004.

[54] M. Kannagi, S. Harada, I. Maruyama, et al., "Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human $CD8^+$ cytotoxic T cells directed against HTLV-I-infected cells," *International Immunology*, vol. 3, no. 8, pp. 761–767, 1991.

[55] S. Jacobson, H. Shida, D. E. McFarlin, A. S. Fauci, and S. Koenig, "Circulating $CD8^+$ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease," *Nature*, vol. 348, no. 6298, pp. 245–248, 1990.

[56] P. K. C. Goon, A. Biancardi, N. Fast, et al., "Human T cell lymphotropic virus (HTLV) type-1-specific $CD8^+$ T cells: frequency and immunodominance hierarchy," *Journal of Infectious Diseases*, vol. 189, no. 12, pp. 2294–2298, 2004.

[57] H. Nose, R. Kubota, N. P. Seth, et al., "Ex vivo analysis of human T lymphotropic virus type 1-specific $CD4^+$ cells by use of a major histocompatibility complex class II tetramer composed of a neurological disease-susceptibility allele and its immunodominant peptide," *Journal of Infectious Diseases*, vol. 196, no. 12, pp. 1761–1772, 2007.

[58] P. K. C. Goon, E. Hanon, T. Igakura, et al., "High frequencies of Th1-type $CD4^+$ T cells specific to HTLV-1 Env and Tax proteins in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis," *Blood*, vol. 99, no. 9, pp. 3335–3341, 2002.

[59] P. K. C. Goon, T. Igakura, E. Hanon, et al., "High circulating frequencies of tumor necrosis factor alpha- and interleukin-2-secreting human T-lymphotropic virus type 1 (HTLV-1)-specific $CD4^+$ T cells in patients with HTLV-1-associated neurological disease," *Journal of Virology*, vol. 77, no. 17, pp. 9716–9722, 2003.

[60] M. Saito, G. P. Taylor, A. Saito, et al., "In vivo selection of T-cell receptor junctional region sequences by HLA-A2 human T-cell lymphotropic virus type 1 Tax11-19 peptide complexes," *Journal of Virology*, vol. 75, no. 2, pp. 1065–1071, 2001.

[61] M. Saito, I. Higuchi, A. Saito, et al., "Molecular analysis of T cell clonotypes in muscle-infiltrating lymphocytes from patients with human T lymphotropic virus type 1 polymyositis," *Journal of Infectious Diseases*, vol. 186, no. 9, pp. 1231–1241, 2002.

[62] C. R. M. Bangham, "The immune response to HTLV-I," *Current Opinion in Immunology*, vol. 12, no. 4, pp. 397–402, 2000.

[63] E. Hanon, S. Hall, G. P. Taylor, et al., "Abundant Tax protein expression in $CD4^+$ T cells infected with human T-cell lymphotropic virus type I (HTLV-I) is prevented by cytotoxic T lymphocytes," *Blood*, vol. 95, no. 4, pp. 1386–1392, 2000.

[64] T. F. Greten, J. E. Slansky, R. Kubota, et al., "Direct visualization of antigen-specific T cells: HTLV-1 Tax11-19-specific $CD8^+$ T cells are activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 13, pp. 7568–7573, 1998.

[65] M. Nagai, R. Kubota, T. F. Greten, J. P. Schneck, T. P. Leist, and S. Jacobson, "Increased activated human T cell lymphotropic virus type I (HTLV-I) Tax11-19-specific memory and effector $CD8^+$ cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis: correlation with HTLV-I provirus load," *Journal of Infectious Diseases*, vol. 183, no. 2, pp. 197–205, 2001.

[66] R. Kubota, T. Kawanishi, H. Matsubara, A. Manns, and S. Jacobson, "Demonstration of human T lymphotropic virus type I (HTLV-I) tax-specific $CD8^+$ lymphocytes directly in peripheral blood of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by intracellular cytokine detection," *Journal of Immunology*, vol. 161, no. 1, pp. 482–488, 1998.

[67] A. H. Sabouri, K. Usuku, D. Hayashi, et al., "Impaired function of human T-lymphotropic virus type 1 (HTLV-1) specific $CD8^+$ T cells in HTLV-1 associated neurologic disease," *Blood*, vol. 112, no. 6, pp. 2411–2420, 2008.

[68] S. E. Macatonia, J. K. Cruickshank, P. Rudge, and S. C. Knight, "Dendritic cells from patients with tropical spastic paraparesis

are infected with HTLV-1 and stimulate autologous lymphocyte proliferation," *AIDS Research and Human Retroviruses*, vol. 8, no. 9, pp. 1699–1706, 1992.

[69] A. Ali, S. Patterson, K. Cruickshank, P. Rudge, A. G. Dalgleish, and S. C. Knight, "Dendritic cells infected in vitro with human T cell leukaemia/lymphoma virus type-1 (HTLV-1); enhanced lymphocytic proliferation and tropical spastic paraparesis," *Clinical and Experimental Immunology*, vol. 94, no. 1, pp. 32–37, 1993.

[70] Y. Itoyama, S. Minato, J. Kira, et al., "Spontaneous proliferation of peripheral blood lymphocytes increased in patients with HTLV-I-associated myelopathy," *Neurology*, vol. 38, no. 8, pp. 1302–1307, 1988.

[71] S. Ijichi, N. Eiraku, M. Osame, et al., "In vitro modulation of lymphocyte proliferation by prednisolone and interferon-$\alpha$ in patients with HTLV-I-associated myelopathy (HAM)," *Journal of Neuroimmunology*, vol. 23, no. 2, pp. 175–178, 1989.

[72] M. Makino, M. Azuma, S.-I. Wakamatsu, et al., "Marked suppression of T cells by a benzothiophene derivative in patients with human T-lymphotropic virus type iassociated myelopathy/tropical spastic paraparesis," *Clinical and Diagnostic Laboratory Immunology*, vol. 6, no. 3, pp. 316–322, 1999.

[73] K. S. Jones, C. Petrow-Sadowski, Y. K. Huang, D. C. Bertolette, and F. W. Ruscetti, "Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of $CD4^+$ T cells," *Nature Medicine*, vol. 14, no. 4, pp. 429–436, 2008.

[74] Y. Yamano, C. J. Cohen, N. Takenouchi, et al., "Increased expression of human T lymphocyte virus type I (HTLV-I) Tax11-19 peptide-human histocompatibility leukocyte antigen A*201 complexes on $CD4^+$ $CD25^+$ T cells detected by peptide-specific, major histocompatibility complex-restricted antibodies in patients with HTLV-I-associated neurologic disease," *Journal of Experimental Medicine*, vol. 199, no. 10, pp. 1367–1377, 2004.

[75] S. L. Manuel, T. D. Schell, E. Acheampong, S. Rahman, Z. K. Khan, and P. Jain, "Presentation of human T cell leukemia virus type 1 (HTLV-1) Tax protein by dendritic cells: the underlying mechanism of HTLV-1-associated neuroinflammatory disease," *Journal of Leukocyte Biology*, vol. 86, no. 5, pp. 1205–1216, 2009.

[76] Y. Koyanagi, Y. Itoyama, N. Nakamura, et al., "In vivo infection of human T-cell leukemia virus type 1 in non-T cells," *Virology*, vol. 196, no. 1, pp. 25–33, 1993.

[77] C. Grant, K. Barmak, T. Alefantis, J. Yao, S. Jacobson, and B. Wigdahl, "Human T cell leukemia virus type I and neurologic disease: events in bone marrow, peripheral blood, and central nervous system during normal immune surveillance and neuroinflammation," *Journal of Cellular Physiology*, vol. 190, no. 2, pp. 133–159, 2002.

[78] M. Abe, F. Umehara, R. Kubota, T. Moritoyo, S. Izumo, and M. Osame, "Activation of macrophages/microglia with the calcium-binding proteins MRP14 and MRP8 is related to the lesional activities in the spinal cord of HTLV-I associated myelopathy," *Journal of Neurology*, vol. 246, no. 5, pp. 358–364, 1999.

[79] M. C. Levin, M. Krichavsky, R. J. Fox, et al., "Extensive latent retroviral infection in bone marrow of patients with HTLV-I-associated neurologic disease," *Blood*, vol. 89, no. 1, pp. 346–348, 1997.

[80] K. Nagafuji, M. Harada, T. Teshima, et al., "Hematopoietic progenitor cells from patients with adult T-cell leukemia-lymphoma are not infected with human T-cell leukemia virus type 1," *Blood*, vol. 82, no. 9, pp. 2823–2828, 1993.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 678648, 9 pages
doi:10.1155/2010/678648

*Research Article*

# *COMT* Val158Met Polymorphism, Executive Dysfunction, and Sexual Risk Behavior in the Context of HIV Infection and Methamphetamine Dependence

**C. A. Bousman,[1,2] M. Cherner,[1] J. H. Atkinson,[1] R. K. Heaton,[1] I. Grant,[1] I. P. Everall,[1] and The HNRC Group[3,4]**

[1] *Department of Psychiatry, University of California San Diego, La Jolla, CA 92103, USA*
[2] *San Diego Joint Doctoral Program in Public Health (Health Behavior), San Diego State/University of California, CA 92182, USA*
[3] *The Naval Hospital, University of California, San Diego, CA 92055, USA*
[4] *The Veterans Affairs San Diego Healthcare System, CA 92161, USA*

Correspondence should be addressed to C. A. Bousman, cbousman@ucsd.edu

Received 18 August 2009; Accepted 10 October 2009

Academic Editor: Marylou V. Solbrig



Catechol-O-methyltransferease (*COMT*) metabolizes prefrontal cortex dopamine (DA), a neurotransmitter involved in executive behavior; the Val158Met genotype has been linked to executive dysfunction, which might increase sexual risk behaviors favoring HIV transmission. Main and interaction effects of *COMT* genotype and executive functioning on sexual risk behavior were examined. 192 sexually active nonmonogamous men completed a sexual behavior questionnaire, executive functioning tests, and were genotyped using blood-derived DNA. Main effects for executive dysfunction but not *COMT* on number of sexual partners were observed. A *COMT* x executive dysfunction interaction was found for number of sexual partners and insertive anal sex, significant for carriers of the Met/Met and to a lesser extent Val/Met genotypes but not Val/Val carriers. In the context of HIV and methamphetamine dependence, dopaminergic overactivity in prefrontal cortex conferred by the Met/Met genotype appears to result in a liability for executive dysfunction and potentially associated risky sexual behavior.

## 1. Introduction

HIV infection is a global pandemic and the population is growing due to successful treatment with highly active antiretroviral therapy (HAART) [1]. Although rates of HIV have been reduced in the United States among most groups as a result of successful public health efforts (e.g., condom accessibility, education programs, media campaigns), sexual risk behavior and subsequent acquisition and/or spread of HIV and other sexually transmitted infections are still of concern among men who have sex with men as well as drug using populations [1]. Thus, it is evident that, despite research and efforts to understand and curb sexual risk behavior within these vulnerable populations, additional work employing novel approaches are needed.

Sexual risk behaviors can be viewed as a composite of numerous behaviors that collectively make-up a complex behavioral phenotype. As with most complex phenotypes, sexual risk behavior is heterogeneous and several factors contribute to the variance that can be observed from one individual to another. To date, a majority of work examining risk factors for sexual risk behavior phenotypes have primarily focused on psychosocial factors (reviewed in [2]) and/or other complex/heterogeneous behavioral phenotypes such as substance use behaviors [3, 4] as indicators for current or future sexual risk behavior. Ultimately these indicators, upon sufficient replication, become candidates for public health interventions that aim to prevent and reduce sexual risk behaviors. However, the trouble with many of these candidates is that they are too proximal to sexual risk behaviors and often cooccur, making it difficult to disentangle temporal precedence and ultimately limit prevention efforts. One relatively novel approach is to examine intermediate phenotypes or endophenotypes [5] such as neurocognitive

factors as well as biological (i.e., genetic) factors. These factors are more distal to the onset of sexual risk behavior and thus are potentially more advantageous candidates for identifying vulnerable individuals and informing prevention efforts for sexual risk behavior.

Studies in literature examining neurocognitive and biological factors as indicators for sexual risk behaviors are limited. In fact, only two studies to date have examined neurocognitive factors [6, 7] and none to our knowledge have examined biological factors as potential indicators. Although this paucity of research is surprising given previous work linking both neurocognitive [8–10] and genetic [11–13] indicators to other health related behaviors, research has established the dopminergic system as a common link between neurocognitive functioning and sexual behavior.

The dopminergic system has been shown to be involved in sexual arousal, motivation and the subsequent rewarding effect of sexual behavior (for detailed review see [14]). Furthermore, DA in the human brain, specifically in the prefrontal cortex (PFC), has been shown to be necessary for proper cognitive functioning to occur and high or low levels of DA in this brain region are known to contribute to individual cognitive differences in humans [15, 16]. The PFC is of particular importance when examining risk behavior in that executive functions such as decision-making, planning, self-monitoring as well as behavior initiation, organization, and inhibition are largely dependent on PFC integrity [17]. Impairment in executive functioning may result in difficulties in assessing relationships between a person's current behavior and future outcomes; thereby resulting in choices and/or responses on the premise of immediate rewards (e.g., pleasure, social acceptance) versus long-term consequences (e.g., viral infections) and an ultimate potential increase in the likelihood for participation in sexual risk behaviors (e.g., unprotected sex, multiple partners) [7, 18]. Thus, mechanisms responsible for maintaining a dopamine balance within the brain and in particular the PFC would appear to be good biological candidates for further exploration of an association between executive dysfunction and sexual risk behavior.

One such candidate is catechol-O-methyltransferease (*COMT*) which is a mammalian enzyme involved in the metabolic degradation of released dopamine, particularly in the PFC [19]. Of particular interest to this study is a common polymorphism involving a Val to Met substitution at codon 158. The Val allele of the *COMT* Val158Met polymorphism is 40% more enzymatically active than the Met allele [20]. Thus, carriers of the Met allele metabolize dopamine at a less efficient rate, resulting in higher levels of dopamine in the synapse and ultimately an escalation in dopamine receptor activation. This differentiation of dopamine receptor activity dependent on *COMT* genotype has led to several investigations into the relationship between *COMT* and executive dysfunction in which the Val allele has been putatively linked to poor performance on executive functioning tasks [21]. However, to our knowledge no work has examined the relationship between *COMT* and sexual risk behavior; albeit studies of similar behaviors such as novelty seeking [22–24], reward dependence [22], as well as affective arousal and regulation [25] have demonstrated significant relationships.

Given the aforementioned paucity of research in the current literature addressing the contribution of genetic and neurocognitive factors on sexual risk behavior, the primary aim of this study was to examine the main effects of executive functioning as well as the main effects of the *COMT* Val158Met polymorphism on sexual risk behavior among a ethnically diverse population of men with and without METH dependence and/or HIV infection. Within this aim, we hypothesized that the highly active *COMT* Val/Val genotype and its putatively associated deficits in executive functioning would be independently associated with sexual risk behaviors. In addition, as a result of previously mentioned research that has demonstrated an association between *COMT* genotype and executive functioning we also explored the potential interaction effects of *COMT* and executive dysfunction on sexual risk behavior.

## 2. Methods

*2.1. Participants.* Participants were volunteers evaluated at the HIV Neurobehavioral Research Center (HNRC) at the University of California in San Diego as part of a cohort study focused on central nervous system effects of HIV and methamphetamine. The current study comprised 192 sexually active non-monogamous men with and without methamphetamine dependence (METH+/−) and/or HIV infection (HIV+/−). Men were classified as non-monogamous if they stated they had "no current partner" at time of assessment. Monogamous men were excluded because unsafe sexual behavior within a monogamous relationship is less risky than in non-monogamous relationships [26].

All participants underwent a comprehensive characterization procedure that included collection of demographic, neuromedical, psychiatric as well as neuropsychiatric information. HIV serological status was determined by enzyme linked immunosorbent assays (ELISA) plus a confirmatory test. Lifetime METH dependence was determined by the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders Version IV (SCID-IV). However, participants were not actively using other substances, with the exception of cannabis and alcohol. Potential participants were excluded if they met lifetime dependence criteria for other drugs, unless the dependence was judged to be remote (more than 5 years ago) and episodic in nature by a doctoral level clinician. Alcohol dependence within the last year was also an exclusion criterion. All participants were seronegative for hepatitis C infection.

Additional information for each participant was collected as it relates to current depressed mood as well as lifetime diagnosis of Major Depression Disorder (MDD) and/or Bipolar Disorder I or II. Current depressed mood was assessed utilizing the Beck Depression Inventory-I (BDI-I) [27] and MDD and Bipolar Disorder were ascertained using the SCID-IV. Information was also collected to determine lifetime dependence on sedatives, cannabis, opioids, cocaine,

hallucinogens, and alcohol, using the SCID-IV. For METH+ participants, additional information was collected regarding age at first use, years of use, and days since last use of METH; whereas for HIV+ participants, HIV RNA plasma copies was ascertained as part of a larger neuromedical evaluation. All participants gave written consent prior to enrollment and all procedures were approved by the Human Research Protection Program of the University of California, San Diego and San Diego State University.

*2.2. Executive Functioning.* Executive functioning was determined as part of a larger comprehensive battery of tests covering seven ability domains (Learning, Memory, Attention/Working Memory, Verbal Fluency, Processing Speed, Abstraction/Problem Solving, and Motor Speed). The executive functioning domain deficit score, of particular focus in this study, was made up of (1) perseverative responses on the Wisconsin Card Sorting Test [28]; (2) errors on the Halstead Category Test [29], which measures abstraction and cognitive flexibility; and (3) time to complete the Trail Making Test part B (Trails B) [30], reflecting ability to switch and maintain attention between ongoing sequences. Raw scores for each of these component tests were converted to demographically-adjusted $T$-scores ($M = 50$, SD = 10), including adjustments for age, education, gender, and ethnicity as available for each test. The demographically-adjusted $T$-scores for each test were then converted into deficit scores, which reflect degree of impairment by setting performances within the normal range at zero with a range from 0 ($T$-score > 39; no impairment) to 5 ($T$-score < 20; severe impairment). Finally, the individual deficit scores were averaged to derive the domain deficit score, which reflects the severity of executive functioning deficit. Previous work has demonstrated that deficit scores achieve good diagnostic agreement with classifications made by blind clinical ratings [31, 32]. All neurocognitive testing and scoring was performed by trained psychometrists blinded to participants' genotypes.

*2.3. Sexual Risk Behavior.* Sexual risk behavior was assessed through an HNRC-developed self-report measure covering the preceding year. Information was gathered with regard to age at first intercourse as well as number of different sex partners. Age at time of first intercourse was coded in years for both male and female partners. However, when two different ages were given for first intercourse, the younger of the two ages was used. In addition, participants were asked to indicate the percentage of time that they used a condom as well as engaged in oral, vaginal, anal (receptive & insertive) and/or intoxicated sex. Responses were recorded on a 6-item, Likert-type scale with a value of 0 = 0%, 1 = 1%–5%, 2 = 6%–25%, 3 = 26%–50%, 4 = 51%–75% and 5 = 76%–100%.

*2.4. DNA Extraction and Genotyping.* DNA was extracted from peripheral blood mononuclear cells stored (three to five years) at −70°C using the QIAamp DNA Mini kit (Qiagen, Valencia, CA; Catalog #51185). The *COMT* Val158Met polymorphism (rs4680) was assayed along with eight other SNPs as part of a concurrent genetic association project at the HNRC. A multiplex PCR technique designed using Sequenom SpectroDESIGNER software (version 3.0.0.3) was employed by inputting a sequence containing 100 bp of flanking sequence on either side of the *COMT* Val158Met polymorphism. The SNP was then grouped into multiplexes so that the extended product would not overlap in mass with any other oligonucleotide present in the reaction mix, and where no primer-primer, primer-product, or non-specific interactions would occur. The PCR was carried out in 384-well reaction plates in a volume of 5 $\mu$l using 10 ng genomic or whole-genome amplified (WGA) DNA. All subsequent steps, up until the reaction, were spotted onto the SpectroCHIP and carried out in the same reaction plate. After PCR, any unincorporated dNTPs from the PCR were removed from the reaction by digestion with Shrimp alkaline phosphatase. dNTPs were removed so that they could not play any role in the extension of the oligonucleotide at the SNP site. The extension reaction was then carried out in the presence of the extension oligonucleotide and a termination mix containing mass-modified dideoxynucleotides which extended the oligonucleotide over the SNP site with one base. Before spotting onto the SpectroCHIP, the reaction was cleaned by incubation with a cation-exchange resin which removed any salts present. The extension product was then spotted onto a 384-well spectroCHIP before being flown in the MALDI-TOF mass spectrometer. Data were collected, in real time, using SpectroTYPER Analyzer 3.3.0.15, SpectraAQUIRE 3.3.1.1 and SpectroCALLER 3.3.0.14 (Sequenom) algorithms. All genotyping was performed by an accredited commercial laboratory (Harvard Medical School-Partners Healthcare Center for Genetics and Genomics, Cambridge, MA CLIA no. 22D1005307).

*2.5. Statistical Analysis.* All statistical tests and procedures were conducted using SPSS 10.0 (SPSS, 2000). Univariate comparisons across the three *COMT* genotypes (i.e., Val/Val, Val/Met, Met/Met) were performed using one-way analysis of variance (ANOVA) for continuous and chi-squared tests for categorical variables. In cases, where data violated normality assumptions medians were calculated and non-parametric tests (i.e., Kruskal-Wallis) performed. To examine the main and explore the interaction effects of executive functioning and *COMT* on sexual risk behaviors, hierarchical multiple linear regressions in accord with Barron and Kenny's approach [33] were conducted for each of the seven sexual risk behaviors (see Section 2.3) under study. Prior to running each analysis, the executive functioning variable was centered and the *COMT* genotype contrast coded to reduce problems resulting from multicollinearity (Kraemer and Blasey, 2004). In addition, interaction terms were created by multiplying *COMT* genotype by the centered executive functioning variable. Next, multiple linear regressions were used to examine potential confounders based on univariate genotype comparisons described above. These confounders included: ethnicity, METH status, HIV status and age at first intercourse. We also included BDI scores based on inclusion of this measure in recent work testing a similar hypothesis

[7]. Results showed that METH status, HIV status, and age at first intercourse accounted for a significant unique variance for all sexual behaviors under investigation ($R^2$ range: 0.06–0.39, $P$s $< .02$). Thus to control for these potential confounding effects, the residuals derived from each of the sexual behavior models were used as the dependent variables for all subsequent regression models. The centered executive functioning variable and *COMT* genotype as well as the new interaction term were then entered as independent variables into seven individual hierarchical multiple regression models using the residuals described above as the dependent variable. For models in which a significant interaction was observed, a final round of regressions were conducted stratified by *COMT* genotype to determine the nature of the interaction between executive functioning and *COMT* on the particular sexual risk behavior. Due to the exploratory nature of the interaction analysiswe selected a relaxed alpha threshold alpha < .10 to reduce Type II errors, albeit the traditional alpha threshold of .05 was used for all other analyses.

## 3. Results

*3.1. Participant Characteristics.* Characteristics of the full sample by each of the three *COMT* genotypes are summarized in Table 1. All three genotype groups were comparable in age, education, sexual behavior, executive functioning, as well as psychiatric and substance dependence histories. However, Val/Val carriers were significantly more likely to identify as African-American ($\chi^2 = 17.67$, $P = .001$), report an earlier age of first intercourse ($F_{(2,189)} = 3.51$, $P = .032$), and be seropositive for HIV ($\chi^2 = 6.57$, $P = .038$). Whereas, Met-carriers (i.e., Met/Met or Val/Met) were significantly more likely to identify as Caucasian ($\chi^2 = 14.32$, $P = .001$). Additionally, among METH+ participants Val/Val carriers reported significantly greater total years of METH use ($F_{(2,87)} = 3.12$, $P = .050$) compared to their Met-carrying counterparts.

*3.2. Main Effects of Executive Functioning and COMT.* Table 2 provides standardized multiple linear regression coefficient estimates for main and interaction effects of executive functioning and *COMT* genotype for each of the seven sexual risk behaviors adjusting for METH status, HIV status, and age at first intercourse. A significant main effect for the executive functioning domain deficit score was observed for number of partners ($\beta = 0.21$, $P = .005$). Additionally, results from the individual executive functioning tests showed a significant main effect for $T$-scores on the Wisconsin Card Sort and Halstead Category tests in adjusted models of oral sex ($\beta = 0.20$, $P = .009$) and condom use ($\beta = -0.16$, $P = .030$), respectively. Main effects were not observed for *COMT* genotype in any of the regression models.

*3.3. Interaction Effects of Executive Functioning and COMT.* Applying an exploratory cut-off of $P < .10$, significant interactions between the executive functioning domain deficit score and *COMT* were observed for number of sexual partners ($\beta = 0.50$, $P = .038$), insertive anal sex ($\beta = 0.50$, $P = .046$), and receptive anal sex ($\beta = 0.50$, $P = .081$) (Table 2). Subsequent stratified analysis by *COMT* genotype, revealed that among carriers of the Met/Met ($\beta = 0.52$, $P = .001$) and to a lesser extent Val/Met ($\beta = 0.20$, $P = .048$) genotype, increases in the executive functioning domain deficit score was significantly associated with increases in the number of sexual partners in the past 12 months. Stratified analysis for insertive and receptive anal sex revealed similar results. Among Met/Met and Val/Met carriers, an increase in executive deficit scores were associated with an increased frequency of insertive (Met/Met: $\beta = 0.18$; Val/Met: $\beta = 0.11$) and receptive (Met/Met: $\beta = 0.13$; Val/Met: $\beta = 0.11$) anal sex in the past 12 months, albeit not statistically significant.

Results of regression analyses to examine interactions between each of the three individual executive functioning tests and *COMT* genotype are also shown in Table 2. For the Wisconsin Card Sort Test no interactions were observed. However, for Trails B, significant interactions with *COMT* were observed for insertive ($\beta = -0.99$, $P = .015$) and receptive ($\beta = -0.75$, $P = .066$) anal sex, as well as oral sex ($\beta = -0.68$, $P = .096$). Stratified regression analysis showed that among carriers of the Met/Met genotype, poor performance on Trails B (i.e., low $T$-scores) was significantly associated with an increased frequency of insertive ($\beta = -0.38$, $P = .028$) but not receptive ($\beta = -0.22$, $P = .225$) anal sex. Interestingly, among carriers of the Val/Val genotype, $T$-scores on Trails B had a significant positive association with oral sex ($\beta = 0.35$, $P = .013$). Finally, for the Halstead Category Test, a single interaction with *COMT* was observed for condom use ($\beta = -1.13$, $P = .006$). Among carriers of the Met/Met ($\beta = -0.49$, $P = .004$) and to a lesser extent Val/Met ($\beta = -0.19$, $P = .064$) genotype, Halstead Category Test $T$-scores were negatively associated with condom use.

## 4. Discussion

To our knowledge this study is the first to examine main effects as well as explore the interaction effects of *COMT* genotype and executive functioning on sexual risk behavior. Our main findings suggest significant executive dysfunction main effects for number of sexual partners as well as frequency of oral sex and condom use. In addition, results of our exploratory interaction analyses provide evidence that *COMT* genotype and executive dysfunction interact in models of number of sexual partners, condom use, insertive and receptive anal sex, as well as oral sex. Stratified analyses further suggest that the strength of these associations is dependent on the number of Met alleles the individual was carrying, with the exception of oral sex in which Val/Val was the informative genotype.

Our significant executive dysfunction main effects for sexual risk behaviors are discordant with the only other study, to our knowledge, that has examined the association between executive dysfunction and sexual risk behavior [7]. In that study, no association was found between executive

Table 1: Characteristics of full sample by COMT genotype.

| | | COMT genotype | | | |
|---|---|---|---|---|---|
| | Full sample ($n = 192$) | Val/Val ($n = 54$) | Val/Met ($n = 103$) | Met/Met ($n = 35$) | |
| Age (years) *M* (sd) | 37 (9) | 35 (9) | 38 (9) | 39 (11) | |
| Education (years) *M* (sd) | 13 (2) | 13 (2) | 13 (2) | 14 (2) | |
| WRAT4 *M* (sd) | 100 (12) | 99 (11) | 100 (12) | 104 (11) | |
| *Ethnicity (row %)* | | | | | |
| Caucasian | 71 | 52 | 78 | 83 | v/v < v/m, m/m** |
| African-American | 15 | 32 | 7 | 11 | v/v > v/m, m/m** |
| Hispanic | 14 | 17 | 16 | 6 | |
| *Executive Functioning Battery* | | | | | |
| Wisconsin card sort test *T* (sd) | 45 (14) | 47 (16) | 44 (13) | 46 (13) | |
| Trials part B *T* (sd) | 49 (11) | 51 (12) | 47 (10) | 52 (11) | |
| Halstead category Test *T* (sd) | 46 (10) | 47 (10) | 44 (10) | 47 (9) | |
| Domain deficit score *M* (sd) | .55 (.69) | .56 (.68) | .62 (.74) | .35 (.47) | |
| Executive impairment (%) | 45 | 46 | 50 | 31 | |
| *Sexual Characteristics/Behavior* | | | | | |
| Age at first intercourse *M* (sd) | 15 (4) | 14 (4) | 16 (4) | 17 (4) | v/v < m/m* |
| Sexual preference (% heterosexual) | 33 | 35 | 31 | 38 | |
| Number partners in past 12 mo *Median* (IQR) | 3 (1,10) | 4 (1, 11) | 3 (1, 10) | 2 (1, 5) | |
| Condom use (>0% in past 12 mo) | 72 | 74 | 71 | 70 | |
| Insertive anal (>0% in past 12 mo) | 62 | 60 | 67 | 52 | |
| Receptive anal (>0% in past 12 mo) | 58 | 60 | 62 | 46 | |
| Oral sex (>0% in past 12 mo) | 93 | 94 | 93 | 94 | |
| Intoxicated sex (>0% in past 12 mo) | 64 | 63 | 66 | 61 | |
| Vaginal sex (>0% in past 12 mo) | 37 | 35 | 35 | 44 | |
| *DSM-IV Psychiatric Disorder (% lifetime)* | | | | | |
| Major depression | 36 | 36 | 35 | 40 | |
| Bipolar I or II | 4 | 8 | 3 | 3 | |
| Beck depression inventory *M* (sd) | 12 (9) | 11 (8) | 13 (10) | 10 (9) | |
| *DSM-IV Substance Dependence (% lifetime)* | | | | | |
| Sedative | 0 | 0 | 0 | 0 | |
| Cannabis | 9 | 9 | 11 | 6 | |
| Opioid | 0 | 0 | 0 | 0 | |
| Cocaine | 7 | 7 | 5 | 14 | |
| Hallucinogen | 0 | 0 | 0 | 0 | |
| Alcohol | 17 | 15 | 20 | 14 | |
| *Methamphetamine Parameters* | | | | | |
| Methamphetamine dependent (%) | 47 | 37 | 52 | 49 | |
| Age at first METH use, yrs *M* (sd) | 24 (9) | 23 (9) | 25 (8) | 27 (10) | |
| Total METH use, yrs *M* (sd) | 11 (6) | 13 (7) | 11 (6) | 8 (4) | v/v > m/m* |
| Last use of METH, days *Median* (IQR) | 91 (36, 274) | 122 (45, 731) | 91 (32, 236) | 91 (30, 244) | |
| *HIV Parameters* | | | | | |
| HIV seropositive (%) | 56 | 70 | 51 | 49 | v/v > v/m, m/m* |
| HIV RNA, plasma (log copies/mL) *M* (sd) | 2.1 (1.9) | 2.4 (1.7) | 2.0 (2.0) | 1.7 (1.9) | |

Table 2: Multivariate linear regression coefficients for main, interaction, and stratified effects of executive functioning and *COMT* in seven sexual risk behavior models.

| | Standardized Beta[a] | | | | | |
|---|---|---|---|---|---|---|
| | Main Effect | | Interaction | Straitfied[b] | | |
| EF measure | EF | COMT | EF × COMT | Val/Val | Val/Met | Met/Met |
| Sexual Risk Behavior Model | ($n = 192$) | ($n = 192$) | ($n = 192$) | ($n = 54$) | ($n = 103$) | ($n = 35$) |
| *Domain Deficit Score* | | | | | | |
| (1) Partners (# past 12 mo) | 0.21** | 0.10 | 0.50** | 0.03 | 0.20** | 0.52*** |
| (2) Condom use (% past 12 mo) | 0.03 | 0.13 | 0.24 | — | — | — |
| (3) Insertive anal (% past 12 mo) | 0.06 | 0.07 | 0.50** | −0.18 | 0.11 | 0.18 |
| (4) Receptive anal (% past 12 mo) | 0.05 | 0.05 | 0.44* | −0.17 | 0.11 | 0.13 |
| (5) Oral sex (% past 12 mo) | −0.10 | 0.07 | 0.40 | — | — | — |
| (6) Intoxicated sex (% past 12 mo) | 0.07 | −0.06 | 0.08 | — | — | — |
| (7) Vaginal sex (% past 12 mo) | −0.03 | −0.04 | −0.28 | — | — | — |
| *Wisconsin Card Sort Test (T-score)* | | | | | | |
| (1) Partners (# past 12 mo) | −0.09 | 0.08 | −0.18 | — | — | — |
| (2) Condom use (% past 12 mo) | −0.10 | 0.12 | −0.25 | — | — | — |
| (3) Insertive anal (% past 12 mo) | 0.01 | 0.07 | −0.31 | — | — | — |
| (4) Receptive anal (% past 12 mo) | −0.02 | 0.04 | −0.38 | — | — | — |
| (5) Oral sex (% past 12 mo) | 0.20** | 0.09 | 0.13 | — | — | — |
| (6) Intoxicated sex (% past 12 mo) | −0.04 | −0.07 | 0.32 | — | — | — |
| (7) Vaginal sex (% past 12 mo) | 0.04 | −0.04 | −0.04 | — | — | — |
| *Trails B (T-score)* | | | | | | |
| (1) Partners (# past 12 mo) | −0.01 | 0.08 | −0.54 | — | — | — |
| (2) Condom use (% past 12 mo) | −0.07 | 0.11 | 0.03 | — | — | — |
| (3) Insertive anal (% past 12 mo) | −0.06 | 0.06 | −0.99** | 0.18 | −0.03 | –0.38** |
| (4) Receptive anal (% past 12 mo) | −0.04 | 0.03 | −0.75* | 0.17 | −0.06 | −0.22 |
| (5) Oral sex (% past 12 mo) | 0.10 | 0.06 | −0.68* | 0.35** | 0.01 | 0.01 |
| (6) Intoxicated sex (% past 12 mo) | −0.11 | −0.07 | 0.13 | — | — | — |
| (7) Vaginal sex (% past 12 mo) | 0.07 | −0.04 | 0.37 | — | — | — |
| *Halstead Category Test (T-score)* | | | | | | |
| (1) Partners (# past 12 mo) | −0.11 | 0.08 | −0.16 | — | — | — |
| (2) Condom use (% past 12 mo) | −0.16** | 0.11 | −1.13** | 0.08 | –0.19* | –0.49*** |
| (3) Insertive anal (% past 12 mo) | 0.01 | 0.05 | −0.22 | — | — | — |
| (4) Receptive anal (% past 12 mo) | −0.01 | 0.03 | −0.59 | — | — | — |
| (5) Oral sex (% past 12 mo) | 0.06 | 0.06 | −0.50 | — | — | — |
| (6) Intoxicated sex (% past 12 mo) | −0.04 | −0.06 | −0.44 | — | — | — |
| (7) Vaginal sex (% past 12 mo) | −0.05 | −0.04 | 0.25 | — | — | — |

[a]all regression models adjusted for METH status, HIV status, age at first intercourse.
[b]stratified analysis of EF effects by genotype was conducted if a significant ($P < .10$) interaction was observed.
EF: executive functioning; COMT: catechol-O-methyltransferease (0 = Val/Val; 1 = Val/Met; 2 = Met/Met)
*$P < .10$; **$P < .05$, ***$P < .005$.

dysfunction and sexual risk behavior among an African-American sample of men and women poly-substance abusers with and without HIV infection. However, three major methodological differences may explain our discordant findings. First, Gonzalez et al. [7] estimated sexual risk behavior in the past 6 months compared to our window of 12 months and also utilized a composite score rather than individual sexual risk behaviors as their dependent variable. Second, executive dysfunction was assessed using the Iowa Gambling Task, delayed non-matching to sample paradigm, and Stroop task-reaction time version which, respectively, measure decision-making, working memory, and response inhibition. Although these tests are well justified, other components of executive functioning such as perseveration, cognitive sequencing, and concept formation which were assessed in the current study, were not examined. Third

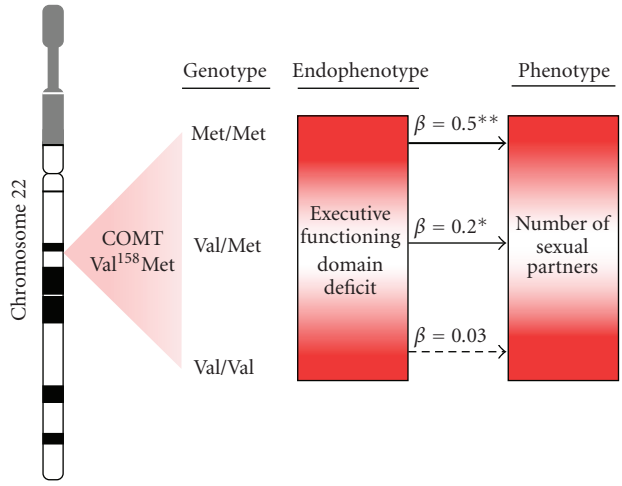



Figure 1: A theoretical model illustrating the interaction effect of a single genotype (i.e., *COMT* Val158Met polymorphism) and endophenotype (i.e., executive functioning domain deficit) on a behavioral phenotype (i.e., number of sexual partners). Among carriers of the *COMT* Met/Met and to a lesser extent the Val/Met genotype the association between executive functioning deficit and number of sexual partners is significantly stronger than among carriers of the Val/Val genotype. $^{*}P < .05$; $^{**}P < .01$.

and finally, regression models were adjusted for sensation seeking, a factor shown in previous research to be associated with sexual risk behavior [34–37]; however, in the current study sensation seeking data was not available and was not adjusted for. Thus, future work examining the association between executive dysfunction and sexual risk behaviors are warranted; particularly research utilizing larger samples with diverse measures of executive functioning and models adjusting for sensation seeking and other personality covariates.

Novel to the current study, we demonstrated several genotype (i.e., *COMT*) by endophenotype (i.e., executive dysfunction) interactions for sexual risk behaviors. A relaxed significance criterion ($P < .10$) produced significant interactions for number of sexual partners, condom use, insertive and receptive anal sex, as well as oral sex. These interactions collectively advocate for further investigation of genotype-endophenotype interactions for sexual risk behavior. However, due to the exploratory nature of these interactions our discussion will be confined to interactions observed for number of sexual partners, frequency of insertive anal sex and condom use, as interactions observed in these models met the traditional significance criterion ($P < .05$).

We observed both a main and interaction effect for number of sexual partners, albeit only within the model including the composite executive functioning deficit score. In this model we found that among carriers of the Met allele (i.e., Met/Met or Val/Met), a positive association between executive functioning deficit and number of sexual partners was present. Thus, among Met allele carriers those with greater deficit scores reported greater number of sexual partners; whereas among Val/Val carriers this association was not significant. Similar to results for number of sexual partners, stratified analysis showed that among carriers of the Met/Met but not Val/Met or Val/Val genotype an positive association between executive dysfunction and frequency of insertive anal sex was present, although only statistically significant for models including the Trails B test. Thus, individuals with lower *T*-scores (i.e., greater impairment) on Trails B reported greater frequency of insertive anal sex only if they were carriers of the Met/Met genotype. Finally, the strongest interaction observed was between *COMT* and the Halstead Category Test for frequency of condom use. Contrary to the expected association, results suggest a negative association among carriers of the Met/Met genotype in which lower *T*-scores (i.e., greater impairment) on the Category Test was associated with an increased frequency of condom use. This unexpected finding may be a result of several factors. First, the psychometric properties of the questionnaire used to measure sexual risk behaviors in our study have not been reported and thus measurement error may be influencing our reported associations. Although there is no agreed upon "gold-standard" for measuring sexual risk behavior, recommendations from a review of 56 sexual risk behavior measures in the literature have been developed [38] and future studies should be encouraged to adopt these measurement strategies to improve accuracy of sexual risk behavior characterization. Second, recall deficits may result in sexual risk behavior reporting errors. This is particularly a concern when measuring sexual risk behavior retrospectively over large spans of time (i.e., 12 months) as was done in the current study. Post-hoc analysis within our sample showed no significant difference in recall deficit by COMT genotype, albeit there did appear to be a trend ($F_{(2,189)} = 2.89$; $P = .058$) in which carriers of the Val/Val genotype had greater deficits than that of Val/Met and Met/Met genotypes (data not shown). Thus, it is possible that recall deficits within the Val/Val group biased our findings toward those in the Met/Met group and should be interpreted with caution. Finally and most speculative, harm reduction campaigns have long aimed to increase condom use within both HIV-infected and METH using populations and our finding may be an artifact of their success.

Collectively, these findings provide a preliminary model of differential susceptibility to sexual risk behavior via executive dysfunction, dependent on *COMT* genotype, particularly the Met/Met genotype (Figure 1). Although the role of the Met/Met genotype is contrary to our hypothesis, our findings, when placed in the context of previous research are informative. Recent research has linked the *COMT* Met/Met genotype to novelty seeking behavior in healthy [39] and methamphetamine using [24] populations. In addition, work by Gonzalez et al. [7] on executive functioning and sexual risk behavior demonstrated that sensation seeking was independently associated with sexual risk, particularly among HIV-seropositive individuals. Thus, it appears that individuals with the Met/Met genotype may have a lower tolerance for monotony and may seek and participate in higher risk behaviors such as METH use or unprotected sex. Furthermore, work by our group and others [40] have suggested that possession of the Met allele enhances executive functioning in healthy controls;

however, this neuroprotective effect is significantly reduced among individuals exposed to methamphetamine. Thus, it is probable that in our sample, of which approximately half were methamphetamine dependent, the putative protective effect of the Met/Met genotype is diminished and propensity to sexual risk behavior enhanced.

It is apparent that the associations between *COMT*, executive dysfunction, and sexual risk behavior are highly complex and context dependent. The current study provides preliminary evidence of these complex relationships and advocates for larger investigations that improve upon and consider several of the limitations that have been presented. Future work should also attempt to address independent and interaction effects of other putative polymorphisms particularly those involved in dopamine synthesis (e.g., Tyrosine Hydroxylase), metabolism (e.g., Monoamine Oxidase A), and reception (e.g., Dopamine Receptors D1-4). In addition, future transdisciplinary work that combines genetic and neurocognitive factors with psychosocial (e.g., trauma, stress) factors will provide valuable insights and elucidate a clearer picture of sexual risk behavior. Completion of such work in combination with the current as well as others previous work will further our understanding of the genotypic and endophenotypic factors involved in the phenotypic expression of sexual risk behaviors and potentially assist with risk identification, prevention, and treatment efforts in the future.

## HNRC Group

The HNRC Group is affiliated with the University of California, San Diego, the Naval Hospital, San Diego, and the Veterans Affairs San Diego Healthcare System, and includes: Director: Igor Grant, M.D.; Codirectors: J. Hampton Atkinson, M.D., Ronald J. Ellis, M.D., Ph.D., and J. Allen McCutchan, M.D.; Center Manager: Thomas D. Marcotte, Ph.D.; Business Manager: Melanie Sherman; Naval Hospital San Diego: Braden R. Hale, M.D., M.P.H. (P.I.); Neuromedical Component: Ronald J. Ellis, M.D., Ph.D. (P.I.), J. Allen McCutchan, M.D., Scott Letendre, M.D., Edmund Capparelli, Pharm.D., Rachel Schrier, Ph.D.; Jennifer Marquie-Beck; Terry Alexander, R.N.; Neurobehavioral Component: Robert K. Heaton, Ph.D. (P.I.), Mariana Cherner, Ph.D., Steven Paul Woods, Psy.D., David J. Moore, Ph.D.; Matthew Dawson, Donald Franklin; Neuroimaging Component: Terry Jernigan, Ph.D. (P.I.), Christine Fennema-Notestine, Ph.D., Sarah L. Archibald, M.A., John Hesselink, M.D., Jacopo Annese, Ph.D., Michael J. Taylor, Ph.D., Neurobiology Component: Eliezer Masliah, M.D. (P.I.), Ian Everall, FRCPsych., FRCPath., Ph.D., Cristian Achim, M.D., Ph.D.; Neurovirology Component: Douglas Richman, M.D., (P.I.), David M. Smith, M.D.; International Component: J. Allen McCutchan, M.D., (P.I.); Developmental Component: Ian Everall, FRCPsych., FRCPath., Ph.D. (P.I.), Stuart Lipton, M.D., Ph.D.; Clinical Trials Component: J. Allen McCutchan, M.D., J. Hampton Atkinson, M.D., Ronald J. Ellis, M.D., Ph.D., Scott Letendre, M.D.; Participant Accrual and Retention Unit: J. Hampton Atkinson, M.D. (P.I.), Rodney von Jaeger, M.P.H.; Data Management Unit: Anthony C. Gamst, Ph.D. (P.I.), Clint Cushman (Data Systems Manager), Daniel R. Masys, M.D. (Senior Consultant); Statistics Unit: Ian Abramson, Ph.D. (P.I.), Florin Vaida, Ph.D., Christopher Ake, Ph.D.

The views expressed in this paper are those of the authors and do not reflect the official policy or position of neither the Department of the Navy, Department of Defense, nor the United States Government.

## References

[1] Joint United Nations Programme on HIV/AIDS, et al., "AIDS epidemic update, 86," 2006.

[2] R. J. Diclemente, C. P. Crittenden, E. Rose, et al., "Psychosocial predictors of HIV-associated sexual behaviors and the efficacy of prevention interventions in adolescents at-risk for HIV infection: what works and what doesn't work?" *Psychosomatic Medicine*, vol. 70, no. 5, pp. 598–605, 2008.

[3] J. D. Fortenberry, "Adolescent substance use and sexually transmitted diseases risk: a review," *Journal of Adolescent Health*, vol. 16, no. 4, pp. 304–308, 1995.

[4] B. C. Leigh and R. Stall, "Substance use and risky sexual behavior for exposure to HIV: issues in methodology, interpretation, and prevention," *American Psychologist*, vol. 48, no. 10, pp. 1035–1045, 1993.

[5] I. I. Gottesman and T. D. Gould, "The endophenotype concept in psychiatry: etymology and strategic intentions," *American Journal of Psychiatry*, vol. 160, no. 4, pp. 636–645, 2003.

[6] A. W. Stacy, M. D. Newcomb, and S. L. Ames, "Implicit cognition and HIV risk behavior," *Journal of Behavioral Medicine*, vol. 23, no. 5, pp. 475–499, 2000.

[7] R. Gonzalez, J. Vassileva, A. Bechara, et al., "The influence of executive functions, sensation seeking, and HIV serostatus on the risky sexual practices of substance-dependent individuals," *Journal of the International Neuropsychological Society*, vol. 11, no. 2, pp. 121–131, 2005.

[8] P. A. Hall, L. J. Elias, and M. Crossley, "Neurocognitive influences on health behavior in a community sample," *Health Psychology*, vol. 25, no. 6, pp. 778–782, 2006.

[9] E. D. Barker, J. R. Séguin, H. R. White, et al., "Developmental trajectories of male physical violence and theft: relations to neurocognitive performance," *Archives of General Psychiatry*, vol. 64, no. 5, pp. 592–599, 2007.

[10] T. R. Barclay, C. H. Hinkin, S. A. Castellon, et al., "Age-associated predictors of medication adherence in HIV-positive adults: health beliefs, self-efficacy, and neurocognitive status," *Health Psychology*, vol. 26, no. 1, pp. 40–49, 2007.

[11] E. W. Triche, N. Hossain, and M. J. Paidas, "Genetic influences on smoking cessation and relapse in pregnant women," *Journal of Obstetrics and Gynaecology*, vol. 28, no. 2, pp. 155–160, 2008.

[12] T. Rankinen and C. Bouchard, "Genetics of food intake and eating behavior phenotypes in humans," *Annual Review of Nutrition*, vol. 26, pp. 413–434, 2006.

[13] M. Tafti, S. Maret, and Y. Dauvilliers, "Genes for normal sleep and sleep disorders," *Annals of Medicine*, vol. 37, no. 8, pp. 580–589, 2005.

[14] M. R. Melis and A. Argiolas, "Dopamine and sexual behavior," *Neuroscience and Biobehavioral Reviews*, vol. 19, no. 1, pp. 19–38, 1995.

[15] A. Nieoullon, "Dopamine and the regulation of cognition and attention," *Progress in Neurobiology*, vol. 67, no. 1, pp. 53–83, 2002.

[16] J. M. Starr, H. Fox, S. E. Harris, I. J. Deary, and L. J. Whalley, "COMT genotype and cognitive ability: a longitudinal aging study," *Neuroscience Letters*, vol. 421, no. 1, pp. 57–61, 2007.

[17] S. W. Anderson and D. Tranel, "Neuropsychological consequences of dysfunction in human dorsolateral prefrontal cortex," in *Handbook of Neuropsychology*, J. Grafman, Ed., pp. 145–156, Elsevier, Amsterdam, The Netherlands, 2002.

[18] A. Bechara, "Risky business: emotion, decision-making, and addiction," *Journal of Gambling Studies*, vol. 19, no. 1, pp. 23–51, 2003.

[19] D. A. Lewis, D. S. Melchitzky, S. R. Sesack, R. E. Whitehead, S. Auh, and A. Sampson, "Dopamine transporter immunoreactivity in monkey cerebral cortex: regional, laminar, and ultrastructural localization," *Journal of Comparative Neurology*, vol. 432, no. 1, pp. 119–136, 2001.

[20] J. Chen, B. K. Lipska, N. Halim, et al., "Functional analysis of genetic variation in catechol-O-methyltransferase (COMT): effects on mrna, protein, and enzyme activity in postmortem human brain," *American Journal of Human Genetics*, vol. 75, no. 5, pp. 807–821, 2004.

[21] J. H. Barnett, P. B. Jones, T. W. Robbins, and U. Müller, "Effects of the catechol-O-methyltransferase Val158Met polymorphism on executive function: a meta-analysis of the Wisconsin Card Sort Test in schizophrenia and healthy controls," *Molecular Psychiatry*, vol. 12, no. 5, pp. 502–509, 2007.

[22] S.-J. Tsai, C.-J. Hong, Y. W.-Y. Yu, and T.-J. Chen, "Association study of catechol-O-methyltransferase gene and dopamine D4 receptor gene polymorphisms and personality traits in healthy young Chinese females," *Neuropsychobiology*, vol. 50, no. 2, pp. 153–156, 2004.

[23] M. Reuter and J. Hennig, "Association of the functional catechol-O-methyltransferase VALI58MET polymorphism with the personality trait of extraversion," *NeuroReport*, vol. 16, no. 10, pp. 1135–1138, 2005.

[24] L. Hosák, J. Libiger, J. Cizek, M. Beránek, and E. Cermáková, "The COMT Val158Met polymorphism is associated with novelty seeking in Czech methamphetamine abusers: preliminary results," *Neuroendocrinology Letters*, vol. 27, no. 6, pp. 799–802, 2006.

[25] E. M. Drabant, A. R. Hariri, A. Meyer-Lindenberg, et al., "Catechol O-methyltransferase val158met genotype and neural mechanisms related to affective arousal and regulation," *Archives of General Psychiatry*, vol. 63, no. 12, pp. 1396–1406, 2006.

[26] L. McKusick, T. J. Coates, S. F. Morin, L. Pollack, and C. Hoff, "Longitudinal predictors of reductions in unprotected anal intercourse among gay men in San Francisco: the AIDS Behavioral Research Project," *American Journal of Public Health*, vol. 80, no. 8, pp. 978–983, 1990.

[27] A. T. Beck, *Depression Causes and Treatment*, University of Pennsylvania Press, Philadelphia, Pa, USA, 1972.

[28] R. K. Heaton and P. Staff, "Wisconsin Card Sorting Test: Computerized Version 2. Research Edition," 1993.

[29] N. A. DeFilippis and P. Staff, "*Category Test: Computer Version*," 1993.

[30] Army Individual Test Battery, *Manual of Directions and Scoring*, Adjunct General's Office, War Deparment, Washington, DC, USA, 1944.

[31] R. K. Heaton, I. Grant, N. Butters, et al., "The HNRC 500—neuropsychology of HIV infection at different disease stages. HIV Neurobehavioral Research Center," *Journal of the International Neuropsychological Society*, vol. 1, no. 3, pp. 231–251, 1995.

[32] C. L. Carey, S. P. Woods, R. Gonzalez, et al., "Predictive validity of global deficit scores in detecting neuropsychological impairment in HIV infection," *Journal of Clinical and Experimental Neuropsychology*, vol. 26, no. 3, pp. 307–319, 2004.

[33] R. M. Baron and D. A. Kenny, "The moderator-mediator variable distinction in social psychological research. Conceptual, strategic, and statistical considerations," *Journal of Personality and Social Psychology*, vol. 51, no. 6, pp. 1173–1182, 1986.

[34] S. C. Kalichman, T. Heckman, and J. A. Kelly, "Sensation seeking as an explanation for the association between substance use and HIV-related risky sexual behavior," *Archives of Sexual Behavior*, vol. 25, no. 2, pp. 141–154, 1996.

[35] S. C. Kalichman, J. R. Johnson, V. Adair, D. Rompa, K. Multhauf, and J. A. Kelly, "Sexual sensation seeking: scale development and predicting AIDS-risk behavior among homosexually active men," *Journal of Personality Assessment*, vol. 62, no. 3, pp. 385–397, 1994.

[36] J. T. Parsons and P. N. Halkitis, "Sexual and drug-using practices of HIV-positive men who frequent public and commercial sex environments," *AIDS Care*, vol. 14, no. 6, pp. 815–826, 2002.

[37] C. S. Hendershot, S. A. Stoner, W. H. George, and J. Norris, "Alcohol use, expectancies, and sexual sensation seeking as correlates of HIV risk behavior in heterosexual young adults," *Psychology of Addictive Behaviors*, vol. 21, no. 3, pp. 365–372, 2007.

[38] S. M. Noar, C. Cole, and K. Carlyle, "Condom use measurement in 56 studies of sexual risk behavior: review and recommendations," *Archives of Sexual Behavior*, vol. 35, no. 3, pp. 327–345, 2006.

[39] V. E. Golimbet, M. V. Alfimova, I. K. Gritsenko, and R. P. Ebstein, "Relationship between dopamine system genes and extraversion and novelty seeking," *Neuroscience and Behavioral Physiology*, vol. 37, no. 6, pp. 601–606, 2007.

[40] V. S. Mattay, T. E. Goldberg, F. Fera, et al., "Catechol O-methyltransferase val158-met genotype and individual variation in the brain response to amphetamine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6186–6191, 2003.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 709791, 6 pages
doi:10.1155/2010/709791

*Review Article*

# Animal Models of CNS Viral Disease: Examples from Borna Disease Virus Models

**Marylou V. Solbrig**

*Department of Internal Medicine (Neurology) and Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada R3A 1R9*

Correspondence should be addressed to Marylou V. Solbrig, msolbrig@uci.edu

Received 1 October 2009; Accepted 8 December 2009

Academic Editor: Guey Chuen Perng



Borna disease (BD), caused by the neurotropic RNA virus, Borna Disease virus, is an affliction ranging from asymptomatic to fatal meningoencephalitis across naturally and experimentally infected warmblooded (mammalian and bird) species. More than 100 years after the first clinical descriptions of Borna disease in horses and studies beginning in the 1980's linking Borna disease virus to human neuropsychiatric diseases, experimentally infected rodents have been used as models for examining behavioral, neuropharmacological, and neurochemical responses to viral challenge at different stages of life. These studies have contributed to understanding the role of CNS viral injury in vulnerability to behavioral, developmental, epileptic, and neurodegenerative diseases and aided evaluation of the proposed and still controversial links to human disease.

## 1. Introduction

Why do we need animal models for neurotropic viral studies? Some day, human functional neuroimaging may provide consistent reliable experimentation in our species, or genetic biomarkers may have predictive validity for disease course or vulnerability. However, until detailed neuroanatomical, molecular and cellular changes needed to understand CNS viral pathogenesis become accessible by these methods, animal models will be needed to evaluate agent-specific and general principles of neurovirology.

In 1985, when Borna disease virus (BDV) was proposed as an etiologic agent of bipolar affective disorder based on detection of BDV antibodies in serum of 1.6% (16/979) of psychiatric patients [1], work on BDV infection of small laboratory animals was well underway. By 1988, when the virus had been implicated in affective disorders and schizophrenia [2], experimental work was expanding and becoming increasingly reliant on in vivo animal studies. Small laboratory animals could be readily infected and demonstrate signs of disease in a short, predictable time frame. Rats and mice offered specific advantages, because rodent nervous systems had been extensively mapped. In time, the use of select animal models would elucidate many neurobiological substrates of viral injury and disrupted behavior, and aid in testing the hypothesis that BDV may be a cause of human neuropsychiatric diseases. The experimental history of use of behaving animals as subjects in Borna disease virus research is the subject of this review.

## 2. History of Borna Disease Virus

Borna disease (BD) was a sporadic, epidemic encephalitis of horses and sheep in 18th and 19th century Central Europe, described as *hitzige Kopfkrankheit* "heated head disease" of cavalry horses in 1885 in the town of Borna in Saxony, Germany [3–5]. In horses, a variety of syndromes were recognized, including excitability and hyperactivity, movement and posture disorders [4, 6, 7]. Although typically fatal, there was sufficient variability in expression and progression of BD to imply that host, age, virus strain, and mode of infection may influence disease. Whether clinical features vary with host attributes or immune status would be followed up by veterinary pathologists and virologists.

Work in experimental BDV infection, begun in rats in the 1960's [8] accelerated in the 1970's and 1980's with more rats, as well as mice, rabbits, tree shrews, and rhesus monkeys. Borna disease had distinct characteristics across species [6]. For example, rats showed hyperactive movements, behavior disorders, and poor maze learning [9–12] while most strains

of mice were asymptomatic [13]. Rhesus monkeys showed hyperactivity, aggression, disinhibited behaviors then apathy [14], and tree shrews, a lower primate, displayed abnormal sexual and social behaviors [15]. For study of BDV, small laboratory animals were becoming an experimentally accessible, less expensive, and reproducible alternative to *post mortem* study of large domestic farm animals.

Early experimental animal work used Strain V, Giessen strain He/80 (Hessen/80) or field strains: wild-type virus from brain suspensions from horses that had recently died. Strain V originated from a naturally infected horse in 1929. Strain V was first prepared as wild-type virus in horse brain homogenate, then developed as a rabbit-adapted BD virus [3] and later rat- or mouse-adapted by serial brain passage in newborn rats or mice [11]. He/80, another horse-derived BDV isolate, was prepared first in rabbits by intracerebral inoculation and cultures of fetal rabbit brain, and then rat- or mouse-adapted by serial brain passage in newborns of the intended experimental species [10].

Not long after reports of an expanding natural host range [5] and demonstration of a broad experimental host range for BDV, came reports of the virus' association with human neuropsychiatric diseases. From the 1985 proposal that Borna disease virus was an etiologic agent of manic-depressive disorders [1], a large number of reports linking Borna disease virus to human affective disorders, schizophrenia, CNS inflammatory and degenerative diseases followed. The associations and controversies regarding human diseases are discussed in several comprehensive reviews [16, 17]. The early associations with human disease drove the development and further expansion of BD animal model systems and testing paradigms. Animal models could be used to address very specific questions, whether Borna disease virus is a cause or cofactor in human neuropsychiatric disease, for example, based on development of serologic, detection and transmission criteria, or recognition of biomarkers of disease.

In addition, the animal models could be used to advance more abstract lines of investigation: whether neurotropic viruses would elucidate aspects of nervous system function and plasticity. Because BDV was associated with psychiatric diseases of man in the late 1980's, diseases with no underlying histopathology by standard microscopy techniques of the time, investigators began to consider whether infection by a non-lytic, slow-growing, neurotropic virus itself could render CNS pharmacologic and neurochemical changes. The background and experimental paradigms for ascertaining the validity of virus-induced neurotransmitter effects were already in place, due to widening knowledge of the functional anatomy, neuropharmacology and neurochemistry of prefrontal, motor, reward, and limbic circuits rendered by animal models developed during the 1980's and 1990's [18]. Animal models for addiction and psychiatry were some of the most robust in neurobiology. The recording of movement, behavior or cognitive disorders in experimentally infected rodents [9–12, 19, 20] and primates [14, 15], led to the study of these syndromes in the context of specific circuit, neurotransmitter or pharmacologic paradigms. In other words, a neural (neurotransmitter) systems approach to solving a problem in viral pathogenesis, such as how BDV causes movement and behavior disorders in affected species, developed. The approach would rely heavily on behavioral testing.

## 3. Adolescent-Infected Rats

In experimentally-infected adolescent rats, BDV caused a multiphasic syndrome characterized by hyperactivity, dyskinesias, stereotypies, excitability, stimulus sensitivity, self-mutilation, followed by dystonia, ataxia, paresis, seizures and premature senescence [9, 19]. Interestingly, the early stage of disease resembled psychostimulant sensitization or overdose in rats. Looking like a syndrome of apparent dopamine (DA) excess or sensitivity, Borna disease of rats could be probed with specific pharmacologic agents to establish the neuropharmacological basis for the behaviors. Behavioral supersensitivity to the dopaminergic psychostimulants d-amphetamine [19] and cocaine [21] was shown using photocell cages for automated activity recordings and behavior rating scales developed for the study of amphetamine dose-response curves in rats.

Demonstration of psychostimulant sensitivity was followed by study of pre- and postsynaptic effects of infection in dopaminergic extrapyramidal motor and reward circuits. In the nigrostriatal system, partial DA deafferentation and compensatory hyperactivity in surviving striatal nerve terminals [19] along with tyrosine hydroxylase (TH) hyperphosphorylation and TH metabolic hyperactivity in nigrostriatal projections [22] were bases for locomotor hyperacitivity and stimulant sensitivity. Reduced dopamine D2 and D3 receptors but preserved D1 receptor numbers in striatum (caudate putamen and nucleus accumbens) [19, 23] underlay the BD rat's motor dyskinesias and later stage dystonia. Reduced D2 striatal receptors and the reduced indirect striatal pathway throughput that results, is the primary pathology also of Huntington's disease, the choreic syndrome of man. Hyperactivity and fearless, disinhibited behaviors were associated with metabolic changes in the prefrontal DA circuits [24], while cognitive decline was linked to forebrain cholinergic loss [25, 26], a pathology of Alzheimer's disease.

The neuropharmacologic and neurochemical changes supported the possibility of a link between BDV and human neuropsychiatric syndromes with dopaminergic substrates, such as schizophrenia, extrapyramidal (movement) disorders, addictions, or cholinergic substrates, such as dementia. Further evidence of neuropharmacologic consequences of infection was provided by successfully treating BD rat abnormal behaviors with the atypical neuroleptic clozapine, or the D1 receptor antagonist SCH23390 [19], which reduces self-mutilation behaviors in Lesch-Nyhan patients.

Since the dopamine system is not mature in adolescent rats, the changes in transmitter levels, synthetic and catabolic enzymes, and receptor numbers described could reflect direct, indirect viral effects, and some of the plastic consequences of early life viral insult. However, the most extensive evaluation of neurodevelopmental consequences of early life viral exposure has been with neonatally infected rats. Lewis rats intracerebrally infected at birth were the first virus-induced Autism-Spectrum Disorders models.

## 4. Neonatal-Infected Rats

Borna disease of neonatally infected rats is a global neurodevelopmental disorder with psychomotor abnormalities, developmental delay, and learning difficulties measured by behavioral tests of reflexes, stance, balance, posture, gait, socialization, learning and play [27–29]. In neonatally-infected animals, the development of neuropathology roughly parallels the timecourse of microglial proliferation and expression of MHC (major histocompatibility complex) class I and class II, ICAM (intercellular adhesion molecule), CD4 and CD8 molecules [30]. Inflammatory, survival, and proapoptotic signals drive remodelling of the brain from early life. Highly plastic systems, such as the monoamines (dopamine, norepinephrine and serotinin) show changes that include reduced serotonin transmission [31–33], an important link to both autism and depression. The anatomic systems that register early life reflexes and programs, such as cerebellum and hippocampus, are permanently changed [28, 32, 34–39]. In fact, in rats, early life infection by BDV without an associated cellular inflammatory response in brain, produces many of the same neuroanatomical, neurochemial, neuroimmune and behavior changes recognized in children with Autism Spectrum Disorders. Significant parallel changes or sequelae include a dysplastic cerebellum, dissolution of the hippocampal dentate gyrus, early life deficits in hippocampal serotonin, and abnormalities in domains of social (play) behavior, emotion (chronic anxiety), and cognition (poor performance on spatial learning and memory) [40].

Whether neonatally infected BD rats specifically model viral effects *in utero* or effects on infant or child development depends on the experimental question and which regions, anatomic features, or behaviors are studied. For example, the birth of the majority of granule cells of dentate gyrus of hippocampus that takes place during first postnatal month in rats, occurs prenatally in man [41]. In this respect, the gross structure of the hippocampus of a newborn rat approximately matches a third trimester human. Therefore, disordered macroscopic development of hippocampus, as complex function of microglia activation [30, 42], neurotrophin and cytokine signaling [28, 43–45], metabolic and oxidative stress ([36, 37, 39], also described in adult infected animals, [46, 47]) has direct relevance to *in utero* virus exposure in man.

Alternatively, other outcome measures have relevance for post-natal or early life consequences of CNS viral exposure or injury. For example, synapse density in the molecular layer of hippocampus reaches adult levels at 21 postnatal days in rat and 7–10 postnatal months in man [41]. Structural causes of disability acquired in infancy would be related to reductions in synaptic growth-associated protein GAP-43, synaptophysin [48], connexin36 [49], and binding of BDV p24 phosphoprotein to neurite growth promoter, amphoterin/HMG-1 [50] found in neonatally infected rats. On the other hand, hippocampal-dependent learning and memory is established 15–16 postnatal days in rat, 4–5 postnatal years in man [41, 51], such that tests of learning can model consequences of viral exposure across a broad span of childhood. Acquisition of spatial and aversive learning, shown to be poor as neonatally infected BD rats mature [12], was associated with increased NPY (neuropeptide Y) expression in hippocampus [52]. Since NPY overexpressed in hippocampus of transgenic rats is associated with spatial learning deficits [53], the results from the BD rats are consistent with NPY upregulation being a viral induced neurochemical cause of compromised learning. The results reflect changes in a cognitive neurotransmitter system that is highly plastic throughout life, contributing to our understanding of neuropeptides in childhood learning.

## 5. Mice

Mice have bridged the gaps between pathogen to behavior and genes to behavior in several important ways. Transgenic technology has enabled studies of the role of individual Borna proteins in disease and molecular genetic mouse models have established mechanisms of host susceptibility and disease outcome.

A transgenic mouse model based on astroglial expression of the BDV phosphoprotein, had reduced BDNF (brain derived neurotrophic factor) levels, serotonin receptor transcripts and synaptic density, and behavioral abnormalities similar to those of neonatal rat infection [54]. Transgenic mice expressing the BDV nucleoprotein in neurons or astrocytes have decreased susceptibility to homotypic infection and disease [55].

Immune determinants of Borna disease have been investigated using genetic and molecular genetic mouse models [56]. Mice, similar to rats, could be infected with BDV by intracerebral inoculation once natural BDV isolates were mouse-adapted by serial passages in rat brain [57]. Most laboratory strains of mice develop persistent infection but remain well [57]. MRL strain mice are an exception, developing meningoencephalitic Borna disease and hyperactive, disinhibited behaviors after adolescent infection [13]. As in rats, severe clinical disease of mice is mediated by MHC class I restricted cytotoxic T cells [58]. Wild type MRL mice infected as neonates develop symptomatic disease mediated by antiviral CD8+ T cells, in contrast to $\beta 2$ microglobulin-knock out MRL mice lacking CD8+ T cells that remain well [58]. In another $\beta 2$ microglobulin-deficient strain, C57BL/10J, also lacking CD8+ T cells, BDV-mice develop persistent non-cytolytic infection with most neurons of hippocampus containing viral antigen. Only mice having high transcript levels of interferon-gamma (IFN-$\gamma$) inducible protein IP-10, a CXC chemokine and chemoattractant for CXCR3+ T cells, showed poor performance on the water maze test of spatial memory and hippocampal function [59, 60]. CNS infection without hippocampal neuronal loss or maldevelopment distinguished neonatal mice from neonatal rat infections, and established a role of IFN-$\gamma$ on behavior and learning when the hippocampus is apparently intact or without structural damage by infection.

Further studies have found differences across species in susceptibility of BDV to the antiviral action of IFN-$\gamma$. Rat IFN-$\gamma$ is less efficient at blocking or reducing infection in

rat cell lines than is human IFN-$\gamma$ at limiting or clearing virus from cells of human and nonhuman primate lineage [61].

## 6. Rats or Mice?

A switch to mice has never been complete. In many practical respects, rats are superior to mice, such as subjects of complex behavior or electroencephalographic studies. Biological strategies for gene silencing, such as small interfering RNA technology, may return some laboratories to the use of rats.

Meanwhile, BD rats can still make contributions to understanding gene $\times$ environment vulnerability questions if the human genetics of a disease are known. For example, epilepsy vulnerability has been associated with a dynorphin promoter region polymorphism, or low dynorphin expression genotype, in man [62]. In animals, the dynorphin system in the hippocampus is known to regulate excitability [63]. The hypothesis that reduced dynorphin expression in the dentate gyrus of hippocampus due to periadolescent virus exposure leads to epileptic responses was successfully tested with adolescent-infected BD rats. Epileptic effects were associated with an absence of dynorphin from the dentate gyrus granule cell layer, thus reproducing a neurochemical marker of epilepsy, namely low dynorphin tone [64].

## 7. Conclusion

Well-chosen animal models can be powerful tools for revealing how CNS viral infection results in disrupted behavior. The methods of behavioral research (use of photocell cages, video tracking, operant measures, EEG and telemetry, developmental ethnograms in home cages and test situations) were not the usual procedures and experimental vocabulary of virus researchers. However, research in BDV has helped to change that. Work on BDV in many labs over 2-3 decades has illustrated the choices of subject and test procedures that yielded important insights into pathogenesis. Work on adolescent-infected rats has shown how infection can cause long-term changes in emotional behavior and cognitive capacity. Work on neonatal rats has shown how infections during brain development can cause long-term changes in behavior and cognitive capacity. Studies on mice have shown infections during brain development can cause long-term changes in these parameters in genetically vulnerable individuals. All together, the studies have shown BDV causes pharmacologic and lesion effects in experimentally infected species with disease outcome related to genetic background and/or developmental maturity of nervous system at the time of viral exposure. The same drugs that treat psychiatric diseases of man treat or suppress the abnormal behaviors of infected rodents. A next step may be to make greater use of models based on genetic variants relevant for neuropsychiatric disorders to study outcomes of infection. It is anticipated that work on BD in animal models will continue to elucidate aspects of nervous system function and disease.

## Acknowledgments

The author's lab has been supported by NIH DA 00376, NS 042307, the Charles and Diane Karp Foundation, and the University of Manitoba Medical Group.

## References

[1] R. Rott, S. Herzog, B. Fleischer, et al., "Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders," *Science*, vol. 228, no. 4700, pp. 755–756, 1985.

[2] L. Bode, S. Riegel, H. Ludwig, J. D. Amsterdam, W. Lange, and H. Koprowski, "Borna disease virus-specific antibodies in patients with HIV infection and with mental disorders," *The Lancet*, vol. 2, no. 8612, p. 689, 1988.

[3] W. Zwick, "Borna'sche krankheit und encephalomyelitis der tiere," in *Handbuch der Viruskrankheiten*, F. Gildemeister, E. Haagen, and O. Waldmann, Eds., vol. 2, pp. 252–354, Fischer, Jena, Germany, 1939.

[4] H. Ludwig and P. Thein, "Demonstration of specific antibodies in the central nervous system of horses naturally infected with Borna disease virus," *Medical Microbiology and Immunology*, vol. 163, no. 4, pp. 215–226, 1977.

[5] R. O. Waelchli, F. Ehrensperger, A. Metzler, and C. Winder, "Borna disease in a sheep," *The Veterinary Record*, vol. 117, no. 19, pp. 499–500, 1985.

[6] H. Ludwig, L. Bode, and G. Gosztonyi, "Borna disease: a persistent virus infection of the central nervous system," *Progress in Medical Virology*, vol. 35, pp. 107–151, 1988.

[7] M. V. Solbrig, J. H. Fallon, and W. I. Lipkin, "Behavioral disturbances and pharmacology of Borna disease," *Current Topics in Microbiology and Immunology*, vol. 190, pp. 93–101, 1995.

[8] E. Nitzschke, "Investigation about the experimental Bornavirus-infection in the rat," *Journal of Veterinary Medicine B*, vol. 10, pp. 470–527, 1963 (German).

[9] O. Narayan, S. Herzog, K. Frese, H. Scheefers, and R. Rott, "Behavioral disease in rats causesd by immunopathological responses to presistent borna virus in the brain," *Science*, vol. 220, no. 4604, pp. 1401–1403, 1983.

[10] O. Narayan, S. Herzog, K. Frese, H. Scheefers, and R. Rott, "Pathogenesis of borna disease in rats: immune-mediated viral ophthalmoencephalopathy causing blindness and behavioral abnormalities," *Journal of Infectious Diseases*, vol. 148, no. 2, pp. 305–315, 1983.

[11] N. Hirano, M. Kao, and H. Ludwig, "Persistent, tolerant or subacute infection in Borna disease virus-infected rats," *Journal of General Virology*, vol. 64, no. 7, pp. 1521–1530, 1983.

[12] W. Dittrich, L. Bode, H. Ludwig, M. Kao, and K. Schneider, "Learning deficiencies in Borna disease virus-infected but clinically healthy rats," *Biological Psychiatry*, vol. 26, no. 8, pp. 818–828, 1989.

[13] S. A. Rubin, R. W. Waltrip II, J. R. Bautista, and K. M. Carbone, "Borna disease virus in mice: host-specific differences in disease expression," *Journal of Virology*, vol. 67, no. 1, pp. 548–552, 1993.

[14] L. Stitz, H. Krey, and H. Ludwig, "Borna disease in rhesus monkeys as a model for uveo-cerebral symptoms," *Journal of Medical Virology*, vol. 6, no. 4, pp. 333–340, 1980.

[15] H. Sprankel, K. Richarz, H. Ludwig, and R. Rott, "Behavior alterations in tree shrews (Tupaia glis, Diard 1820) induced by Borna disease virus," *Medical Microbiology and Immunology*, vol. 165, no. 1, pp. 1–18, 1978.

[16] W. I. Lipkin and T. Briese, "Bornaviridae," in *Field's Virology*, D. M. Knipe and P. M. Howley, Eds., vol. 2, pp. 1829–1851, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 5th edition, 2007.

[17] APMIS, "From animals to man—50 years of development. Proceedings of the International Berlin Symposium on Bornavirus Infections," *APMIS. Supplementum*, no. 124, pp. 3–97, 2008.

[18] M. V. Solbrig and G. F. Koob, "Neuropharmacological sequelae of persistent CNS viral infections: lessons from Borna disease virus," *Pharmacology Biochemistry and Behavior*, vol. 74, no. 4, pp. 777–787, 2003.

[19] M. V. Solbrig, G. F. Koob, J. H. Fallon, and W. I. Lipkin, "Tardive dyskinetic syndrome in rats infected with Borna disease virus," *Neurobiology of Disease*, vol. 1, no. 3, pp. 111–119, 1994.

[20] G. Gosztonyi and H. Ludwig, "Borna disease—neuropathology and pathogenesis," *Current Topics in Microbiology and Immunology*, vol. 190, pp. 39–73, 1995.

[21] M. V. Solbrig, G. F. Koob, and W. I. Lipkin, "Cocaine sensitivity in Borna disease virus-infected rats," *Pharmacology Biochemistry and Behavior*, vol. 59, no. 4, pp. 1047–1052, 1998.

[22] M. V. Solbrig, G. F. Koob, L. H. Parsons, et al., "Neurotrophic factor expression after CNS viral injury produces enhanced sensitivity to psychostimulants: potential mechanism for addiction vulnerability," *The Journal of Neuroscience*, vol. 20, article RC104, pp. 1–5, 2000.

[23] M. V. Solbrig, G. F. Koob, J. N. Joyce, and W. I. Lipkin, "A neural substrate of hyperactivity in Borna disease: changes in brain dopamine receptors," *Virology*, vol. 222, no. 2, pp. 332–338, 1996.

[24] M. V. Solbrig, G. F. Koob, J. H. Fallon, S. Reid, and W. I. Lipkin, "Prefrontal cortex dysfunction in Borna disease virus (BDV)-infected rats," *Biological Psychiatry*, vol. 40, no. 7, pp. 629–636, 1996.

[25] U. Gies, T. Bilzer, L. Stitz, and J. F. Staiger, "Disturbance of the cortical cholinergic innervation in Borna disease prior to encephalitis," *Brain Pathology*, vol. 8, no. 1, pp. 39–48, 1998.

[26] U. Gies, T. J. Görcs, J. Mulder, et al., "Cortical cholinergic decline parallels the progression of Borna virus encephalitis," *NeuroReport*, vol. 12, no. 17, pp. 3767–3772, 2001.

[27] J. R. Bautista, G. J. Schwartz, J. C. de la Torre, T. H. Moran, and K. M. Carbone, "Early and persistent abnormalities in rats with neonatally acquired Borna disease virus infection," *Brain Research Bulletin*, vol. 34, no. 1, pp. 31–40, 1994.

[28] M. Hornig, H. Weissenböck, N. Horscroft, and W. I. Lipkin, "An infection-based model of neurodevelopmental damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 21, pp. 12102–12107, 1999.

[29] M. V. Pletnikov, T. H. Moran, and K. M. Carbone, "Borna disease virus infection of the neonatal rat: developmental brain injury model of autism spectrum disorders.," *Frontiers in Bioscience*, vol. 7, pp. D593–D607, 2002.

[30] H. Weissenböck, M. Hornig, W. F. Hickey, and W. I. Lipkin, "Microglial activation and neuronal apoptosis in bornavirus infected neonatal lewis rats," *Brain Pathology*, vol. 10, no. 2, pp. 260–272, 2000.

[31] M. V. Pletnikov, S. A. Rubin, G. J. Schwartz, K. M. Carbone, and T. H. Moran, "Effects of neonatal rat Borna disease virus (BDV) infection on the postnatal development of the brain monoaminergic systems," *Developmental Brain Research*, vol. 119, no. 2, pp. 179–185, 2000.

[32] M. V. Pletnikov, S. A. Rubin, M. W. Vogel, T. H. Moran, and K. M. Carbone, "Effects of genetic background on neonatal Borna disease virus infection-induced neurodevelopmental damage—part II: neurochemical alterations and responses to pharmacological treatments," *Brain Research*, vol. 944, no. 1-2, pp. 108–123, 2002.

[33] D. M. Dietz, M. W. Vogel, S. A. Rubin, T. H. Moran, K. M. Carbone, and M. V. Pletnikov, "Developmental alterations in serotoninergic neurotransmission in Borna disease virus (BDV)-infected rats: a multidisciplinary analysis," *Journal of Neurovirology*, vol. 10, no. 5, pp. 267–277, 2004.

[34] L. M. Eisenman, R. Brothers, M. H. Tran, et al., "Neonatal Borna disease virus infection in the rat causes a loss of Purkinje cells in the cerebellum," *Journal of Neurovirology*, vol. 5, no. 2, pp. 181–189, 1999.

[35] M. V. Pletnikov, S. A. Rubin, T. H. Moran, and K. M. Carbone, "Exploring the cerebellum with a new tool: neonatal Borna disease virus (BDV) infection of the rat's brain," *Cerebellum*, vol. 2, no. 1, pp. 62–70, 2003.

[36] B. L. Williams and W. I. Lipkin, "Endoplasmic reticulum stress and neurodegeneration in rats neonatally infected with borna disease virus," *Journal of Virology*, vol. 80, no. 17, pp. 8613–8626, 2006.

[37] B. L. Williams, K. Yaddanapudi, C. M. Kirk, A. Soman, M. Hornig, and W. I. Lipkin, "Metallothioneins and zinc dysregulation contribute to neurodevelopmental damage in a model of perinatal viral infection," *Brain Pathology*, vol. 16, no. 1, pp. 1–14, 2006.

[38] B. L. Williams, K. Yaddanapudi, M. Hornig, and W. I. Lipkin, "Spatiotemporal analysis of purkinje cell degeneration relative to parasagittal expression domains in a model of neonatal viral infection," *Journal of Virology*, vol. 81, no. 6, pp. 2675–2687, 2007.

[39] B. L. Williams, M. Hornig, K. Yaddanapudi, and W. I. Lipkin, "Hippocampal poly(ADP-ribose) polymerase 1 and caspase 3 activation in neonatal bornavirus infection," *Journal of Virology*, vol. 82, no. 4, pp. 1748–1758, 2008.

[40] M. V. Pletnikov, M. L. Jones, S. A. Rubin, T. H. Moran, and K. M. Carbone, "Rat model of autism spectrum disorders: genetic background effects on borna disease virus-induced developmental brain damage," *Annals of the New York Academy of Sciences*, vol. 939, pp. 318–319, 2001.

[41] S. Avishai-Eliner, K. L. Brunson, C. A. Sandman, and T. Z. Baram, "Stressed-out, or in (utero)?" *Trends in Neurosciences*, vol. 25, no. 10, pp. 518–524, 2002.

[42] M. V. Ovanesov, K. Moldovan, K. Smith, M. W. Vogel, and M. V. Pletnikov, "Persistent Borna Disease Virus (BDV) infection activates microglia prior to a detectable loss of granule cells in the hippocampus," *Journal of Neuroinflammation*, vol. 5, article 16, 2008.

[43] C. Sauder and J. C. de La Torre, "Cytokine expression in the rat central nervous system following perinatal Borna disease virus infection," *Journal of Neuroimmunology*, vol. 96, no. 1, pp. 29–45, 1999.

[44] C. R. Plata-Salamán, S. E. Ilyin, D. Gayle, A. Romanovitch, and K. M. Carbone, "Persistent Borna disease virus infection of neonatal rats causes brain regional changes of mRNAs for cytokines, cytokine receptor components and neuropeptides," *Brain Research Bulletin*, vol. 49, no. 6, pp. 441–451, 1999.

[45] C. Sauder, W. Hallensleben, A. Pagenstecher, et al., "Chemokine gene expression in astrocytes of Borna disease virus-infected rats and mice in the absence of inflammation," *Journal of Virology*, vol. 74, no. 19, pp. 9267–9280, 2000.

[46] D. C. Hooper, S. T. Ohnishi, R. Kean, Y. Numagami, B. Dietzschold, and H. Koprowski, "Local nitric oxide production in viral and autoimmune diseases of the central nervous system,"

*Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 12, pp. 5312–5316, 1995.

[47] D. C. Hooper, R. B. Kean, G. S. Scott, et al., "The central nervous system inflammatory response to neurotropic virus infection is peroxynitrite dependent," *Journal of Immunology*, vol. 167, no. 6, pp. 3470–3477, 2001.

[48] D. Gonzalez-Dunia, M. Watanabe, S. Syan, M. Mallory, E. Masliah, and J. C. de La Torre, "Synaptic pathology in Borna disease virus persistent infection," *Journal of Virology*, vol. 74, no. 8, pp. 3441–3448, 2000.

[49] C. Köster-Patzlaff, S. M. Hosseini, and B. Reuss, "Loss of connexin36 in rat hippocampus and cerebellar cortex in persistent Borna disease virus infection," *Journal of Chemical Neuroanatomy*, vol. 37, no. 2, pp. 118–127, 2009.

[50] W. Kamitani, Y. Shoya, T. Kobayashi, et al., "Borna disease virus phosphoprotein binds a neurite outgrowth factor, amphoterin/HMG-1," *Journal of Virology*, vol. 75, no. 18, pp. 8742–8751, 2001.

[51] A. Diamond, "Rate of maturation of the hippocampus and the developmental progression of children's performance on the delayed non-matching to sample and visual paired comparison tasks," *Annals of the New York Academy of Sciences*, vol. 608, pp. 394–433, 1990.

[52] C. R. Plata-Salamán, S. E. Ilyin, D. Gayle, A. Romanovitch, and K. M. Carbone, "Persistent Borna disease virus infection of neonatal rats causes brain regional changes of mRNAs for cytokines, cytokine receptor components and neuropeptides," *Brain Research Bulletin*, vol. 49, no. 6, pp. 441–451, 1999.

[53] A. Thorsell, M. Michalkiewicz, Y. Dumont, et al., "Behavioral insensitivity to restraint stress, absent fear suppression of behavior and impaired spatial learning in transgenic rats with hippocampal neuropeptide Y overexpression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 23, pp. 12852–12857, 2000.

[54] W. Kamitani, E. Ono, S. Yoshino, et al., "Glial expression of Borna disease virus phosphoprotein induces behavioral and neurological abnormalities in transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 8969–8974, 2003.

[55] M. Rauer, J. Götz, D. Schuppli, P. Staeheli, and J. Hausmann, "Transgenic mice expressing the nucleoprotein of Borna disease virus in either neurons or astrocytes: decreased susceptibility to homotypic infection and disease," *Journal of Virology*, vol. 78, no. 7, pp. 3621–3632, 2004.

[56] J. Hausmann, W. Hallensleben, J. C. de La Torre, et al., "T cell ignorance in mice to Borna disease virus can be overcome by peripheral expression of the viral nucleoprotein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 17, pp. 9769–9774, 1999.

[57] M. Kao, H. Ludwig, and G. Gosztonyi, "Adaptation of Borna disease virus to the mouse," *Journal of General Virology*, vol. 65, no. 10, pp. 1845–1849, 1984.

[58] W. Hallensleben, M. Schwemmle, J. Hausmann, et al., "Borna disease virus-induced neurological disorder in mice: infection of neonates results in immunopathology," *Journal of Virology*, vol. 72, no. 5, pp. 4379–4386, 1998.

[59] C. Sauder, W. Hallensleben, A. Pagenstecher, et al., "Chemokine gene expression in astrocytes of Borna disease virus-infected rats and mice in the absence of inflammation," *Journal of Virology*, vol. 74, no. 19, pp. 9267–9280, 2000.

[60] C. Sauder, D. P. Wolfer, H.-P. Lipp, P. Staeheli, and J. Hausmann, "Learning deficits in mice with persistent Borna disease virus infection of the CNS associated with elevated chemokine expression," *Behavioural Brain Research*, vol. 120, no. 2, pp. 189–201, 2001.

[61] C. Sauder, I. Herpfer, C. Hässler, and P. Staeheli, "Susceptibility of Borna disease virus to the antiviral action of gamma-interferon: evidence for species-specific differences," *Archives of Virology*, vol. 149, no. 11, pp. 2171–2186, 2004.

[62] E. Stögmann, A. Zimprich, C. Baumgartner, S. Aull-Watschinger, V. Höllt, and F. Zimprich, "A functional polymorphism in the prodynorphin gene promotor is associated with temporal lobe epilepsy," *Annals of Neurology*, vol. 51, no. 2, pp. 260–263, 2002.

[63] M. Simonato and P. Romualdi, "Dynorphin and epilepsy," *Progress in Neurobiology*, vol. 50, no. 5-6, pp. 557–583, 1996.

[64] M. V. Solbrig, R. Adrian, J. Baratta, J. C. Lauterborn, and G. F. Koob, "Kappa opioid control of seizures produced by a virus in an animal model," *Brain*, vol. 129, no. 3, pp. 642–654, 2006.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 380456, 10 pages
doi:10.1155/2010/380456

*Review Article*

# Animal Models of Virus-Induced Neurobehavioral Sequelae: Recent Advances, Methodological Issues, and Future Prospects

**Marco Bortolato and Sean C. Godar**

*Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA*

Correspondence should be addressed to Marco Bortolato, bortolat@usc.edu

Received 15 August 2009; Revised 14 November 2009; Accepted 9 March 2010

Academic Editor: Marylou V. Solbrig



Converging lines of clinical and epidemiological evidence suggest that viral infections in early developmental stages may be a causal factor in neuropsychiatric disorders such as schizophrenia, bipolar disorder, and autism-spectrum disorders. This etiological link, however, remains controversial in view of the lack of consistent and reproducible associations between viruses and mental illness. Animal models of virus-induced neurobehavioral disturbances afford powerful tools to test etiological hypotheses and explore pathophysiological mechanisms. Prenatal or neonatal inoculations of neurotropic agents (such as herpes-, influenza-, and retroviruses) in rodents result in a broad spectrum of long-term alterations reminiscent of psychiatric abnormalities. Nevertheless, the complexity of these sequelae often poses methodological and interpretational challenges and thwarts their characterization. The recent conceptual advancements in psychiatric nosology and behavioral science may help determine new heuristic criteria to enhance the translational value of these models. A particularly critical issue is the identification of intermediate phenotypes, defined as quantifiable factors representing single neurochemical, neuropsychological, or neuroanatomical aspects of a diagnostic category. In this paper, we examine how the employment of these novel concepts may lead to new methodological refinements in the study of virus-induced neurobehavioral sequelae through animal models.

## 1. The Role of Viruses in Psychiatric Disorders

The implication of viruses in the pathogenesis of neuropsychiatric disorders has been posited since the foundations of modern psychiatry. Few years after the outbreak of the 1918 flu pandemic, the nosographic analogies between postinfluenza encephalitic manifestations and psychotic traits prompted Menninger [1] to conjecture an infective etiology for some subtypes of schizophrenia. Although this hypothesis sank into relative obsolescence during the following decades, the identification of lentiviruses rekindled enthusiasm for the viral theory of mental disorders and laid the foundations of psychovirology as a novel discipline [2]. Over the last 25 years, numerous studies have shown that viral infections of the central nervous system are potentially conducive to profound behavioral and cognitive perturbations. Typical outcomes of viral encephalitis, for instance, encompass impairments in sensory reactivity and information processing, emotional lability, and sleep disturbances. Notably, the severity of some postencephalitic manifestations is often correlated to the degree of morphological and functional damage induced by both the viral infection and/or the ensuing immune response [3]. A detailed analysis of these phenomena is beyond the scope of this paper and can be found elsewhere [4, 5].

Neuropsychiatric sequelae of overt infectious processes, while accounting for few documented cases of mental illness, offer a compelling theoretical platform to postulate the role of viruses in the pathophysiology of schizophrenia and other neurodevelopmental syndromes. According to the prevalent theories, the pathogenesis of these disorders may be initiated or contributed by early exposure to low-virulence, neurotropic viruses. The resulting infection, albeit subclinical, may interfere with key neurogenetic processes and lead to insidious, potentially ingravescent functional impairments. Specifically, this neuropathic process has been

shown to reflect the combination of the insults mediated by the cytopathic and mutagenic effects of the virus [6], as well as the immune response [7–10]. Furthermore, the chronic enactment of compensatory, neuroprotective responses may also contribute to the pathogenesis of these disorders. For example, certain clusters of psychotic manifestations may result from the outcome of the hippocampal dysregulation by virus-mediated and endogenous anticonvulsant mechanisms [11]. The confluence of these processes with neurodevelopmental mechanisms and other factors of vulnerability (both genetic and environmental) may result in severe neurochemical imbalances, brain architectural irregularities, and mental derangement.

The implication of viruses in mental disorders is supported by several lines of epidemiological evidence. Prenatal or perinatal viral infections are risk factors for schizophrenia and autism-spectrum disorders [12, 13]. Numerous epidemiological surveys have also documented a birth excess of schizophrenia and bipolar patients in winter and spring [14–17] or seasonal correlations between incidence of autism-spectrum disorders and viral outbreaks [18–22].

The viral hypothesis of mental disorders is also corroborated by clinical evidence. Several investigations have shown the presence of viral antigens and antibodies in plasma or post-mortem samples of mental patients [23–25]. In particular, these studies have revealed that schizophrenia may be associated with several families of viruses, including herpes viruses [26–30], orthomyxoviruses [31–35], and retroviruses [36, 37]. It is worth noting that most of these agents have also been associated with autism-spectrum disorders [38, 39], bipolar disorder [40–42], and other psychiatric conditions [43].

However, there remain numerous inconsistencies across different clinical studies concerning the role of viruses in psychiatric disorders. The lack of reproducible associations may be contingent on different parameters related to the virus (rarity, reproductive cycle, virulence, latency), host (age at the time of infection, genetic vulnerability, immune responsiveness) and infective process (modality of transmission, duration and severity of the infection, patterns of viral replication and distribution within the host, viral interaction with other infective agents and environmental factors). Other discrepancies may reflect methodological limitations in the design and execution of the studies, including imprecise diagnostic assessments, poor sensitivity and specificity of survey criteria, insufficient number of subjects, low statistical power, and lack of adequate controls.

## 2. Animal Models of Virus-Induced Neurobehavioral Sequelae

*2.1. Choice of Animal.* Animal models provide a powerful tool to explore the biological substrates of virus-induced neuropsychiatric sequelae in a controlled experimental setting. The best target to address specific pathophysiological hypotheses is afforded by mammalian species, and in particular nonhuman primates, in view of their relatively high degree of anatomical and phylogenetic continuity with humans. Ethical considerations, however, dictate that experimentation on monkeys should be strictly limited to exceptional circumstances, where no viable alternatives are available, such as the research on simian immunodeficiency virus as a neuroAIDS model. Rodents offer an attractive compromise for research on viral neurobehavioral disorders, by virtue of their advantageous characteristics, including small size, short reproductive cycle, and cost-effectiveness. The inherent differences between humans and rodent species in the key factors of pathogenesis of virus-induced sequelae—such as neurodevelopmental mechanisms and immune reactivity—are an important limiting factor in the application of these models. These shortcomings notwithstanding, rodent models have a high level of validity, which is warranted by common physiological, neurobiological, and ethological characteristics. Indeed, investigations on mice (*Mus musculus*) and rats (*Rattus norvegicus*) have become the richest source of knowledge on the neural substrates and molecular underpinnings of post-infectious behavioral dysregulations. These models have been instrumental to the acquisition of a large body of information on virus-induced neurobehavioral alterations.

A thorough overview of the body of evidence on the specific virus-induced neurobehavioral alterations is covered in the excellent reviews by Tomonaga [5] and Weed and Gold [44]. In the following sections, we will focus on the key conceptual issues and advances in rodent models of virus-induced neurobehavioral disorders and indicate new potential experimental directions to reduce confounding factors in the analysis of these abnormalities.

*2.2. Validity.* Rodent models of virus-induced neurobehavioral alterations are a striking example of *homologous models*, aimed at reproducing the etiology, pathophysiology and symptomatic presentation of psychiatric disorders. In substantial agreement with the classical criteria of validity for animal models [45], the degree of isomorphism between virus-induced sequelae and the corresponding psychiatric should be assessed at the following four levels:

(i) etiological validity, based on the epidemiological and/or clinical relevance of the pathogen agents to the targeted disorder;

(ii) face validity, describing the analogies between the virus-induced behavioral manifestations of the animal model and the signs and symptoms of the targeted psychiatric disorder;

(iii) predictive validity, signifying the responsiveness of the model to clinically efficacious treatments (such as antipsychotic agents for schizophrenia etc.);

(iv) construct validity, representing the pathophysiological congruence between animal and human neurobiological alterations.

A prominent case of fulfillment of the above standards is illustrated by the outcomes of prenatal and/or early postnatal exposure to influenza virus as a model of schizophrenia:

(i) the etiological validity of the model is strongly supported by the aforementioned epidemiological

association between influenza and psychotic disorders;

(ii) the face validity of the model is ensured by the resemblance between the symptoms of schizophrenia and the array of behavioral changes observed in the animals, including alterations in exploratory activity, social behavior, emotional reactivity, gating, sleep patterns, working, and spatial memory [46–51];

(iii) the predictive validity of the model is based on its responsiveness to atypical antipsychotic agents [50];

(iv) the construct validity of the model is warranted by the implication of some of the key areas in the pathophysiology of schizophrenia, such as neocortex and hippocampus [52, 53], as well as by the demonstration of alterations in line with those shown in these disorders, such as alterations of pyramidal neurons, glia, and myelination processes [52–59].

Another example is represented by rodents subjected to early HSV inoculation, which exhibit an array of schizophrenia-related deficits, including preattentional and cognitive impairments [60–63] and alterations of the serotonergic system and hippocampus [64, 65].

Although the adoption of stringent validity criteria ensures high sensitivity and specificity of preclinical findings, caution should be exercised in their utilization. In particular, over-reliance on the use of reference compounds as gold standards for predictive validity—such as conventional antipsychotic agents for models of virus-induced psychotic disorders—has been shown to impoverish the translational potential of numerous models [66, 67]. Furthermore, the etiological validity of these models is inherently tempered by the impossibility to reproduce specific virus-host interactions. In view of these considerations, several lines of research have recently focused on the identification of relevant behavioral manifestations and pathophysiological alterations induced by neurotropic viruses, irrespective of their proven implication of human pathology. This concept is clearly exemplified by experimentation on borna-disease virus (BDV), a single-stranded RNA virus whose association with in behavioral disorders is well-established in animals, yet still highly controversial in humans [68, 69]. BDV infection triggers a broad spectrum of abnormalities in animals, which encompass emotional impairments, aggressiveness, stereotyped behavior, and cognitive deficits [70, 71]. These alterations are supported by neurochemical changes in dopamine and neurotrophin signaling [71–76]. These and other studies of viral effects on neurotransmitter, neurohormonal, neurotrophic systems, and inflammatory mediators have represented a useful template to study the mechanisms of virus- and immune-mediated behavioral disorders, generalizable to other viral and pathogen agents.

*2.3. Intermediate Phenotypes and Endophenotypes.* A critical complication posed by models of psychiatric disorders is based on the absence of biomarkers and other quantitative indices in the current criteria of classification outlined in the Diagnostic and Statistical Manual of Mental Disorders (DMS) IV TR. This absence results in a mismatch between the diagnostic and the pathophysiological perspectives. Nosologic categories such as schizophrenia, for example, are merely based on limited symptomatic and diagnostic criteria and may be broad rubrics encompassing a number of heterogeneous disorders with overlapping semeiological features. Conversely, conditions with similar etiology and pathophysiology may be set apart by artificial diagnostic criteria. In this perspective, viral sequelae may only represent specific subsets of mental disorders.

The acknowledgement of this scenario has fostered a lively academic debate on the reorganization of translational principles in psychiatry and reiterated the necessity of incorporating pathophysiological criteria in the classification of mental disorders [67]. This discussion has led to the implementation of quantitative, pathophysiological, and dimensional criteria in the new conceptual guidelines for the next edition of the DSM (DSM-V) [77–79].

An important corollary of this theoretical reorganization of translational science has been the development of the concept of *intermediate phenotypes.* These quantifiable factors represent neuroanatomical, biochemical, neurophysiological, neuropsychological, or cognitive traits, reflecting a simpler architecture than the whole array of abnormalities featured in a diagnostic category [80]. An important variant of this notion is that of *endophenotype,* defined as a heritable intermediate phenotype which, albeit not inherently pathological, is an element of vulnerability to mental disorders [81, 82]. The validation of a biological characteristic as an endophenotype is dictated by a set of criteria that reflect and emphasize its genetic nature, including cosegregation in nonaffected relatives of the probands [83, 84].

A typical example of a well-established neuropsychological endophenotype is the prepulse inhibition (PPI) of the startle reflex. PPI is the reduction in startle response elicited by a strong sensory stimulus that occurs when the latter is preceded by a weaker signal [85]. This parameter is considered a highly dependable index for the measurement of gating function, and its deficits are typically observed in schizophrenia, bipolar disorder, autism, and other disorders [86]. Notably, PPI deficits are not only featured by mental patients, but also by their nonaffected relatives [87, 88].

Although the manifestations of endophenotypes may become apparent only in correspondence of specific environmental contingencies, they are always active and independent from the symptomatic fluctuations of the associated disorder. Furthermore, endophenotypes are generally not pathognomonic of specific mental disorders, but they are often featured in different diagnostic categories, probably signifying common pathophysiological mechanisms between disorders.

Endophenotypes have recently garnered increasing popularity in psychiatry, as they offer a suitable operational appendix to the body of knowledge derived from the characterization of human genome and generation of multiple lines of transgenic rodents. Furthermore, this strategy may be vital for the progress in our understanding of neurodevelopmental disorders [89–93]. The dissection of complex behavioral syndromes into more elementary "building blocks"—such as

quantifiable responses related to specific neuropsychological traits—is more amenable to effective translational strategies and may lead to a profitable exchange of information between human and animal studies and the identification of common indices in mental patients and animal models [67, 94, 95].

Analogous considerations can be applied to the case of virus-derived neurobehavioral abnormalities. In particular, the identification of intermediate phenotypes in virus-based animal models is critical to simplify the mapping of their abnormalities, elucidate pathophysiological links, and enhance the translational value of these experimental preparations. In parallel with the criteria postulated for the definition of endophenotypes in behavioral genetics, virus-dependent intermediate phenotypes could be theorized as outcomes of viral (or immune) action on specific neurobiological substrates. These neurochemical, morphological, or psychological traits should reflect specific factors of vulnerability to a neurodevelopmental disorder, but not overt pathological manifestations. Accordingly, they should also be observed in mental patients as well as in non-affected individuals with an overlapping history of early viral infection (or at least analogous antibody panels, signifying similar antigenic history).

The identification of these targets in humans, however, is extremely arduous, in view of the high variability of natural conditions affecting the infectious process and its outcomes. Thus, high-quality characterization of animal models is indispensable to delineate future strategies on clinical targets. To this end, the design of behavioral studies should place particular emphasis on quantitative, dimensional, and time-specific relations between face and construct validity. For instance, experiments on viral sequelae should take into account the behavioral and cognitive properties associated with the brain areas of viral tropism. Furthermore, the analysis of virus-induced disorders should be performed across different time points, to capture the progressive changes induced by the ongoing virus-mediated insult (through neurotoxic proteins and oxidative stress), immune responses (through cytokine release, microglial activation, etc.), neurodevelopmental processes, and neural compensatory mechanisms. For example, this conceptual framework has been employed to study the time-related effects of maternal influenza on cortical, hippocampal, and cerebellar morphogenesis [53, 96].

*2.4. Experimental Design and Analysis: Tackling Problems of Inconsistency and Reproducibility.* The experimental design related to the phenotyping of virus-induced sequelae is based on an extremely complex algorithm, which reflects a top-down progression from generic, multimodal paradigms towards increasingly specific tasks for the measurement of subtle traits. This approach is instrumental for the deconstructing strategy used for the identification of intermediate phenotypes.

As previously mentioned, the evolving state of the disorder dictates the need for a meticulous temporal documentation of the course of viral pathogenesis, which should be accomplished through periodic examinations of the infected rodents during their major developmental milestones. A general protocol for the assessment of neurobehavioral sequelae of experimental infections is outlined in Table 1. The phenotypic assessment should typically include auxological (size, body weight, etc.), physiological (heart rate, breathing frequency, body temperature, and food and water intake), and neurological parameters (posture, gait, motor coordination, reflex integrity, sensory perception, and pain sensitivity) [97]. Impairments in these functions may call for specific adjustments of the experimental design and testing procedure. For example, acoustic alterations may require paradigms relying on alternative sensory modalities, such as visual or tactile. Similarly, deficits in locomotor and cardiorespiratory functions may justify the selection of smaller arenas for behavioral testing and shorter experimental duration (to avoid fatigue). Finally, observations should include a brief ethological analysis of nesting, grooming, exploratory, and social behaviors, and so forth. This preliminary monitoring can be critical to refine hypotheses on potential behavioral impairments to test in the experimental phase.

The identification of intermediate behavioral phenotypes induced by early viral infection should be performed with a battery of subsequent tests. The operational procedure starts with the recognition of behavioral differences between virus-infected rodents and their controls in highly standardized paradigms, such as the open field. Alterations of the performance in this apparatus can inform on numerous components of behavioral and cognitive repertoire, such as locomotion, exploration, spontaneous activity, anxiety-like responses, behavioral rigidity, and memory (upon repeated exposure to the apparatus) [98]. The spectrum of open-field behaviors directs the selection of new paradigms to further qualify the nature of the observed variations. Subsequent adjustments towards progressively narrower targets reshape the architecture of the decisional flowchart and allow the detection of specific intermediate phenotypes. Testing conditions should always be optimized in view of the specific aspects of the experimentation, to capture potential variations with the highest sensitivity. In this context, environmental variations (such as light and noise level) in the experimental setting, as well as the employment of pharmacological agents can be extremely valuable to reveal hidden non-apparent traits and elucidate underlying neurobiological mechanisms.

As no single assay can completely model a psychiatric phenotype, conclusions based on a limited set of paradigms can prove detrimental to the analysis and lead to false positive (or negative) results. Instead, characterization of phenotypes should be based on a multimodal approach with complementary paradigms that can capture different facets of behavioral domains. More generally, experimental strategies aimed at the identification of virus-induced intermediate phenotypes should always be multifactorial and employ several complementary measures (including non-behavioral indices, such as neurochemical, electrophysiological, and physiological parameters). The documentation of coherent indications from multiple variables ensures the translational quality and the construct validity of the findings.

Table 1: Experimental protocol for phenotyping of neurobehavioral sequelae of experimental infections in rodents.

| |
|---|
| Preliminary physical assessment: |
| Auxological parameters (body size, weight, growth rate, dentition, and sexual development) |
| Physiological parameters (heart rate, breathing frequency, rectal temperature, food and water daily intake, and menstrual cycle) |
| Presence of pathological features (non-experimental infections, fur loss, ringtail, chromodacryorrhea, adynamic ileus, neoplasms, etc.) |
| Neurological assessment: |
| Sensory acuity and discrimination (vision, hearing, olfaction, taste, vibrissae activity and somatosensation, and pain sensitivity) |
| Motor assessment (posture, orienting, prehension, gait, motor coordination, reflex integrity, Arousal and sleep patterns) |
| Ethological assessment of spontaneous behavior: |
| Home-cage behavior (locomotion, nesting, etc.) |
| Exploratory activity (novelty responsiveness and foraging behavior) |
| Social behavior (playing behavior, aggressive and defensive responses, mating, maternal behavior) |
| Grooming behavior (syntactical and nonchain) |
| Age-specific behaviors (suckling and huddling in pups, food reaching and social play in adolescent rodents, etc.) |
| Presence of abnormal behaviors (stereotyped behaviors and maladaptive reactivity to stimuli, etc.) |
| Standardized behavioral assays: |
| Multimodal assays (open field, object exploration, etc.) |
| Domain-specific assays |

One of the most vexing and insidious aspects of behavioral research on virus-induced sequelae is the low reproducibility of findings across different laboratories and settings. This problem is generally ascribed to a large contingent of numerous unpredictable and irreducible environmental variables that cannot be fully accounted for in behavioral testing. Low reproducibility undermines the progress of behavioral neuroscience and the improvement of translational strategies, calling for urgent strategies to counter this problem.

One of the key strategies to maximize reproducibility in behavioral research is the standardization of experimental procedures. Nevertheless, this approach has been recently challenged [99], in light of the discrepant findings yielded by behavioral experiments performed with identical protocols in different laboratories. These authors have suggested an alternative approach to reduce intergroup variance, consisting in the heterogenization of experimental conditions.

Despite the persuasiveness of some of the arguments used to justify this strategy, standardization of certain conditions remains an essential requisite for some factors, such as gender and age. Furthermore, it is worth noting that intermediate phenotypes are defined as factors of vulnerability that are unraveled only in association with select genetic or environmental conditions; thus, insufficient standardization of conditions such as strain, husbandry, and testing conditions may temper the ability of a given paradigm to characterize and identify novel targets. This possibility is particularly concerning in reference to the analysis of virus-derived intermediate phenotypes. In fact, alterations induced by viral perturbation of a neural pathway may not result in full pathological processes, but may only confer a liability to a disorder. As this condition may be revealed only in presence of specific genetic or environmental factors, experimental heterogenization may impoverish the heuristic value of animal testing. These premises justify the need for stringent standardization throughout all the experimental phases of the study. In particular, extreme care should be exercised in the standardization of the following aspects:

(i) inoculation protocol: this standardization is critical to minimize apparent and predictable factors of variability, such as the characteristics of the host (strain, gender, age, etc.), the virus (strain, virulence, tropism), and the infection procedure (infectious dosage, regimen and route of administration). This latter concept is particularly important, as experimental infections are generally performed *in utero* or during critical stages of early postnatal neurodevelopment, which are characterized by the occurrence of numerous time-sensitive morphogenetic processes;

(ii) environmental conditions of husbandry and experimental testing: this prescription is essential to control (and preferably minimize) potential sources of stress. Indeed, non-viral stress interferes with the pathophysiological trajectory of virus-induced behavioral sequelae and is a major confounder in behavioral analysis. Stress-induced secretion of glucocorticoids leads to inhibition of cell immunity and reactivation of latent viruses [100–102]. Stress can also modulate immune functions through other hormones (including corticotrophin-releasing hormone, growth hormone, and prolactin) and neurotransmitters, such as catecholamines [103] and serotonin [104]. This aspect can become extremely critical with models of virus-induced sequelae that have been shown to alter monoamine signaling, such as influenza virus and BDV [59, 71, 74, 105, 106].

The reliability and translational impact of the findings can also be enhanced with a thorough statistical approach, which should not only be restricted to comparisons between

cases and controls based on ANOVA or its nonparametric counterparts. Temporal analysis of behavior can be fundamental to illustrate the progression of virus-induced alterations. Alternatively, correlational approaches can be employed to link particular behavioral outcomes to specific variations in antibody panel, neurotransmitter levels, morphology, electrophysiological alterations, drug sensitivity, or other changes in molecular substrates.

Data analysis can also be improved by the elimination of statistical artifacts, such as floor or ceiling effects, which can be overcome by the incorporation of alternative methods of analysis. Another typical example of artifact is offered by the "litter effect", defined as the tendency for littermates to exhibit similar responses to the same early insult. This potential pitfall can generate spurious statistical effects and should always be avoided by limiting the number of tested animals within each litter or by adjusting the statistical analysis of the behavioral data to account for kinship within the same treatment group.

Finally, a higher degree of replicability may be ensured by the adoption of different criteria for the rejection of null hypotheses. Indeed, the establishment of a $P$ level of .05 as a threshold for "statistical significance" has been extensively criticized [107, 108] and is accepted only on account of historical reasons [109]. However, the researcher should take into account that the employment of extremely stringent statistical conventions may lead to substantial drawbacks, such as the reduction of the positive predictive value and the heuristic validity of experimental procedures.

In conclusion, the design of any experiment aimed at behavioral phenotyping of virus-induced neurobehavioral sequelae should always take into account a number of key issues:

(i) definition of meaningful endpoints, based on intermediate phenotypes revealed by cross-species paradigms (testable in both humans and animals);
(ii) environmental standardization and elimination of exogenous stressors;
(iii) analysis at different time points, to capture the progression of viral pathogenesis;
(iv) correlational analysis between behavioral indices and other paradigms, to strengthen the construct validity of the animal model.

## 3. Concluding Remarks and Future Directions

Research on virus-induced neurobehavioral sequelae is advancing as new methodological developments in translational strategies and behavioral sciences set a promising scenario for future advancements. The field of phenotypic characterization is at a critical juncture and has recently witnessed the introduction of wide-range approaches, such as those afforded by transcriptomics, proteomics, metabonomics, and epigenetics. These techniques can provide a rich source of candidate traits for the identification of intermediate phenotypes [110, 111]. Furthermore, these approaches can identify patterns of a specific "viral signature", which may help facilitate the diagnosis and the link with a viral etiology without requiring the analysis of viral material in numerous clinical specimens [112–114].

The establishment of a neurophenomic approach to psychiatric diagnosis and classification with extensive profiling of affective and cognitive traits through batteries of psychometric scales [115, 116] is bound to stimulate the development of parallel guidelines and establish a sound foundation for improvements in experimental design on animal models, with a particular emphasis on construct validity criteria. This perspective should strengthen our understanding of the neural mechanisms underlying the role of viruses in psychiatric disorders and accelerate the identification of therapeutic targets.

## References

[1] K. A. Menninger, "Influenza and schizophrenia. An analysis of post-influenzal "dementia precox," as of 1918, and five years later further studies of the psychiatric aspects of influenza," *American Journal of Psychiatry*, vol. 82, no. 6, pp. 469–529, 1926.

[2] C. A. Kaufmann, J. R. Stevens, and E. F. Torrey, "1983 World Health Organization symposium on psychovirology," *Archives of General Psychiatry*, vol. 41, no. 12, pp. 1184–1185, 1984.

[3] F. L. Wilkie, K. Goodkin, M. H. Van Zuilen, M. D. Tyll, R. Lecusay, and T. Edwin, "Cognitive effects of HIV-1 infection," *CNS Spectrums*, vol. 5, no. 5, pp. 33–51, 2000.

[4] A. H. Mohammed, E. Norrby, and K. Kristensson, "Viruses and behavioural changes: a review of clinical and experimental findings," *Reviews in the Neurosciences*, vol. 4, no. 3, pp. 267–286, 1993.

[5] K. Tomonaga, "Virus-induced neurobehavioral disorders: mechanisms and implications," *Trends in Molecular Medicine*, vol. 10, no. 2, pp. 71–77, 2004.

[6] G. J. Atkins, I. M. Balluz, G. M. Glasgow, et al., "Analysis of the molecular basis of neuropathogenesis of RNA viruses in experimental animals: relevance for human disease?" *Neuropathology and Applied Neurobiology*, vol. 20, no. 2, pp. 91–102, 1994.

[7] M. Dalod, T. P. Salazar-Mather, L. Malmgaard, et al., "Interferon $\alpha/\beta$ and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo," *Journal of Experimental Medicine*, vol. 195, no. 4, pp. 517–528, 2002.

[8] M. A. Muñoz-Fernández and M. Fresno, "The role of tumour necrosis factor, interleukin 6, interferon-$\gamma$ and inducible nitric oxide synthase in the development and pathology of the nervous system," *Progress in Neurobiology*, vol. 56, no. 3, pp. 307–340, 1998.

[9] J. S. Orange and C. A. Biron, "An absolute and restricted requirement for IL-12 in natural killer cell IFN-$\gamma$ production and antiviral defense: studies of natural killer and T cell responses in contrasting viral infections," *Journal of Immunology*, vol. 156, no. 3, pp. 1138–1142, 1996.

[10] T. P. Salazar-Mather, T. A. Hamilton, and C. A. Biron, "A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense," *Journal of Clinical Investigation*, vol. 105, no. 7, pp. 985–993, 2000.

[11] Marco Bortolato and Marylou V. Solbrig, "The price of seizure control: dynorphins in interictal and postictal

psychosis," *Psychiatry Research*, vol. 151, no. 1-2, pp. 139–143, 2007.

[12] G. R. DeLong, S. C. Bean, and F. R. Brown, "Acquired reversible autistic syndrome in acute encephalopathic illness in children," *Archives of Neurology*, vol. 38, no. 3, pp. 191–194, 1981.

[13] D. A. Lewis, "Retroviruses and the pathogenesis of schizophrenia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4293–4294, 2001.

[14] J. H. Boyd, A. E. Pulver, and W. Stewart, "Season of birth: schizophrenia and bipolar disorder," *Schizophrenia bulletin*, vol. 12, no. 2, pp. 173–186, 1986.

[15] E. H. Hare, J. S. Price, and E. Slater, "Schizophrenia and season of birth," *British Journal of Psychiatry*, vol. 120, no. 554, pp. 124–125, 1972.

[16] R. A. Machon, S. A. Mednick, and F. Schulsinger, "The interaction of seasonality, place of birth, genetic risk and subsequent schizophrenia in a high risk sample," *British Journal of Psychiatry*, vol. 143, no. 4, pp. 383–388, 1983.

[17] E. F. Torrey, B. B. Torrey, and M. R. Peterson, "Seasonality of schizophrenic births in the United States," *Archives of General Psychiatry*, vol. 34, no. 9, pp. 1065–1070, 1977.

[18] B. D. Bartlik, "Monthly variation in births of autistic children in North Carolina," *Journal of the American Medical Women's Association*, vol. 36, no. 12, pp. 363–368, 1981.

[19] E. Fombonne, "Season of birth and childhood psychosis," *British Journal of Psychiatry*, vol. 155, pp. 655–661, 1989.

[20] C. Gillberg, "Do children with autism have March birthdays?" *Acta Psychiatrica Scandinavica*, vol. 82, no. 2, pp. 152–156, 1990.

[21] M. M. Konstantareas, P. Hauser, C. Lennox, and S. Homatidis, "Season of birth in infantile autism," *Child Psychiatry and Human Development*, vol. 17, no. 1, pp. 53–65, 1986.

[22] Y. Tanoue, S. Oda, F. Asano, and K. Kawashima, "Epidemiology of infantile autism in southern Ibaraki, Japan: differences in prevalence in birth cohorts," *Journal of Autism and Developmental Disorders*, vol. 18, no. 2, pp. 155–166, 1988.

[23] D. J. Hart, R. G. Heath, F. J. Sautter, et al., "Antiretroviral antibodies: implications for schizophrenia, schizophrenia spectrum disorders, and bipolar disorder," *Biological Psychiatry*, vol. 45, no. 6, pp. 704–714, 1999.

[24] S. Srikanth, V. Ravi, K. S. Poornima, K. T. Shetty, B. N. Gangadhar, and N. Janakiramaiah, "Viral antibodies in recent onset, nonorganic psychoses: correspondence with symptomatic severity," *Biological Psychiatry*, vol. 36, no. 8, pp. 517–521, 1994.

[25] H. Terayama, Y. Nishino, M. Kishi, K. Ikuta, M. Itoh, and K. Iwahashi, "Detection of anti-Borna Disease Virus (BDV) antibodies from patients with schizophrenia and mood disorders in Japan," *Psychiatry Research*, vol. 120, no. 2, pp. 201–206, 2003.

[26] S. L. Buka, M. T. Tsuang, E. F. Torrey, M. A. Klebanoff, D. Bernstein, and R. H. Yolken, "Maternal infections and subsequent psychosis among offspring," *Archives of General Psychiatry*, vol. 58, no. 11, pp. 1032–1037, 2001.

[27] S. L. Buka, T. D. Cannon, E. F. Torrey, and R. H. Yolken, "Maternal exposure to herpes simplex virus and risk of psychosis among adult offspring," *Biological Psychiatry*, vol. 63, no. 8, pp. 809–815, 2008.

[28] F. Dickerson, B. Kirkpatrick, J. Boronow, C. Stallings, A. Origoni, and R. Yolken, "Deficit schizophrenia: association with serum antibodies to cytomegalovirus," *Schizophrenia Bulletin*, vol. 32, no. 2, pp. 396–400, 2006.

[29] J. J. Kim, B. H. Shirts, M. Dayal, et al., "Are exposure to cytomegalovirus and genetic variation on chromosome 6p joint risk factors for schizophrenia?" *Annals of Medicine*, vol. 39, no. 2, pp. 145–153, 2007.

[30] E. F. Torrey, M. F. Leweke, M. J. Schwarz, et al., "Cytomegalovirus and schizophrenia," *CNS Drugs*, vol. 20, no. 11, pp. 879–885, 2006.

[31] C. E. Barr, S. A. Mednick, and P. Munk-Jorgensen, "Exposure to influenza epidemics during gestation and adult schizophrenia. A 40-year study," *Archives of General Psychiatry*, vol. 47, no. 9, pp. 869–874, 1990.

[32] T. D. Cannon, S. A. Mednick, J. Parnas, F. Schulsinger, J. Praestholm, and A. Vestergaard, "Developmental brain abnormalities in the offspring of schizophrenic mothers. I. Contributions of genetic and perinatal factors," *Archives of General Psychiatry*, vol. 50, no. 7, pp. 551–564, 1993.

[33] S. A. Mednick, R. A. Machon, M. O. Huttunen, and D. Bonett, "Adult schizophrenia following prenatal exposure to an influenza epidemic," *Archives of General Psychiatry*, vol. 45, no. 2, pp. 189–192, 1988.

[34] E. O'Callaghan, P. Sham, N. Takei, G. Glover, and R. M. Murray, "Schizophrenia after prenatal exposure to 1957 A2 influenza epidemic," *Lancet*, vol. 337, no. 8752, pp. 1248–1250, 1991.

[35] N. Takei, P. Sham, E. O'Callaghan, G. K. Murray, G. Glover, and R. M. Murray, "Prenatal exposure to influenza and the development of schizophrenia: is the effect confined to females?" *American Journal of Psychiatry*, vol. 151, no. 1, pp. 117–119, 1994.

[36] H. Karlsson, S. Bachmann, J. Schröder, J. McArthur, E. F. Torrey, and R. H. Yolken, "Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4634–4639, 2001.

[37] H. Perron, L. Mekaoui, C. Bernard, F. Veas, I. Stefas, and M. Leboyer, "Endogenous retrovirus type W GAG and envelope protein antigenemia in serum of schizophrenic patients," *Biological Psychiatry*, vol. 64, no. 12, pp. 1019–1023, 2008.

[38] B. Anlar, F. Oktem, and T. Torok, "Human parvovirus B19 antibodies in infantile autism," *Journal of Child Neurology*, vol. 9, no. 1, pp. 104–105, 1994.

[39] E. Y. Deykin and B. MacMahon, "Original contributions. Viral exposure and autism," *American Journal of Epidemiology*, vol. 109, no. 6, pp. 628–638, 1979.

[40] A. D. Allen, H. H. Fudenberg, and R. E. Allen, "Affective disorder and viral infections," *Archives of General Psychiatry*, vol. 44, no. 8, p. 760, 1987.

[41] L. E. DeLisi, J. S. Nurnberger, L. R. Goldin, S. Simmons-Alling, and E. S. Gershon, "Epstein-Barr virus and depression," *Archives of General Psychiatry*, vol. 43, no. 8, pp. 815–816, 1986.

[42] E. F. Torrey, R. H. Yolken, and C. J. Winfrey, "Cytomegalovirus antibody in cerebrospinal fluid of schizophrenic patients detected by enzyme immunoassay," *Science*, vol. 216, no. 4548, pp. 892–894, 1982.

[43] J. G. Millichap, "Etiologic classification of attention-deficit/hyperactivity disorder," *Pediatrics*, vol. 121, no. 2, pp. e358–e365, 2008.

[44] M. R. Weed and L. H. Gold, "Paradigms for behavioral assessment of of viral pathogenesis," *Advances in Virus Research*, vol. 56, pp. 583–626, 2001.

[45] P. Willner, "Validation criteria for animal models of human mental disorders: learned helplessness as a paradigm case," *Progress in Neuropsychopharmacology and Biological Psychiatry*, vol. 10, no. 6, pp. 677–690, 1986.

[46] L. Asp, S. Beraki, K. Kristensson, S. O. Ögren, and H. Karlsson, "Neonatal infection with neurotropic influenza A virus affects working memory and expression of type III Nrg1 in adult mice," *Brain, Behavior, and Immunity*, vol. 23, no. 6, pp. 733–741, 2009.

[47] L. Asp, M. Holtze, S. B. Powell, H. Karlsson, and S. Erhardt, "Neonatal infection with neurotropic influenza A virus induces the kynurenine pathway in early life and disrupts sensorimotor gating in adult Tap1-/- mice," *International Journal of Neuropsychopharmacology*. In press.

[48] S. Beraki, F. Aronsson, H. Karlsson, S. O. Ögren, and K. Kristensson, "Influenza A virus infection causes alterations in expression of synaptic regulatory genes combined with changes in cognitive and emotional behaviors in mice," *Molecular Psychiatry*, vol. 10, no. 3, pp. 299–308, 2005.

[49] C. A. Conn, J. L. McClellan, H. F. Maassab, C. W. Smitka, J. A. Majde, and M. J. Kluger, "Cytokines and the acute phase response to influenza virus in mice," *American Journal of Physiology*, vol. 268, no. 1, pp. R78–R84, 1995.

[50] L. Shi, S. H. Fatemi, R. W. Sidwell, and P. H. Patterson, "Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring," *Journal of Neuroscience*, vol. 23, no. 1, pp. 297–302, 2003.

[51] L. A. Toth, J. E. Rehg, and R. G. Webster, "Strain differences in sleep and other pathophysiological sequelae of influenza virus infection in naive and immunized mice," *Journal of Neuroimmunology*, vol. 58, no. 1, pp. 89–99, 1995.

[52] S. H. Fatemi, E. S. Emamian, R. W. Sidwell, et al., "Human influenza viral infection in utero alters glial fibrillary acidic protein immunoreactivity in the developing brains of neonatal mice," *Molecular Psychiatry*, vol. 7, no. 6, pp. 633–640, 2002.

[53] S. H. Fatemi, J. Earle, R. Kanodia, et al., "Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: implications for genesis of autism and schizophrenia," *Cellular and Molecular Neurobiology*, vol. 22, no. 1, pp. 25–33, 2002.

[54] D. Cotter, N. Takei, M. Farrell, et al., "Does prenatal exposure to influenza in mice induce pyramidal cell disarray in the dorsal hippocampus?" *Schizophrenia Research*, vol. 16, no. 3, pp. 233–241, 1995.

[55] S. H. Fatemi, E. S. Emamian, D. Kist, et al., "Defective corticogenesis and reduction in Reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice," *Molecular Psychiatry*, vol. 4, no. 2, pp. 145–154, 1999.

[56] S. H. Fatemi, R. Sohr, G. Juckel, et al., "Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders," *Schizophrenia Research*, vol. 99, no. 1–3, pp. 56–70, 2008.

[57] S. H. Fatemi, T. D. Folsom, T. J. Reutiman, et al., "Abnormal expression of myelination genes and alterations in white matter fractional anisotropy following prenatal viral influenza infection at E16 in mice," *Schizophrenia Research*, vol. 112, no. 1–3, pp. 46–53, 2009.

[58] L. Shi, S. E. P. Smith, N. Malkova, D. Tse, Y. Su, and P. H. Patterson, "Activation of the maternal immune system alters cerebellar development in the offspring," *Brain, Behavior, and Immunity*, vol. 23, no. 1, pp. 116–123, 2009.

[59] C. Winter, T. J. Reutiman, T. D. Folsom, et al., "Dopamine and serotonin levels following prenatal viral infection in mouse-implications for psychiatric disorders such as schizophrenia and autism," *European Neuropsychopharmacology*, vol. 18, no. 10, pp. 712–716, 2008.

[60] David R. Beers, Jenny S. Henkel, Raymond P. Kesner, and William G. Stroop, "Spatial recognition memory deficits without notable CNS pathology in rats following herpes simplex encephalitis," *Journal of the Neurological Sciences*, vol. 131, no. 2, pp. 119–127, 1995.

[61] L. S. Crnic and L. I. Pizer, "Behavioral effects of neonatal herpes simplex type 1 infection of mice," *Neurotoxicology and Teratology*, vol. 10, no. 4, pp. 381–386, 1988.

[62] J. A. Engel, J. Zhang, T. Bergström, et al., "Neonatal herpes simplex virus type 1 brain infection affects the development of sensorimotor gating in rats," *Brain Research*, vol. 863, no. 1-2, pp. 233–240, 2000.

[63] D. J. McFarland and J. Hotchin, "Host genetics and the behavioral sequelae to herpes encephalitis in mice," *Physiology and Behavior*, vol. 30, no. 6, pp. 881–884, 1983.

[64] E. Lycke and B. E. Roos, "Virus infection in infant mice causing persistent impairment of turnover of brain catecholamines," *Journal of the Neurological Sciences*, vol. 26, no. 1, pp. 49–60, 1975.

[65] H. M. Wu, C. C. Huang, S. H. Chen, et al., "Herpes simplex virus type 1 inoculation enhances hippocampal excitability and seizure susceptibility in mice," *European Journal of Neuroscience*, vol. 18, no. 12, pp. 3294–3304, 2003.

[66] C. Belzung, "The genetic basis of the pharmacological effects of anxiolytics: A review based on rodent models," *Behavioural Pharmacology*, vol. 12, no. 6-7, pp. 451–460, 2001.

[67] A. Markou, C. Chiamulera, M. A. Geyer, M. Tricklebank, and T. Steckler, "Removing obstacles in neuroscience drug discovery: the future path for animal models," *Neuropsychopharmacology*, vol. 34, no. 1, pp. 74–89, 2009.

[68] J. M. Pyper, "Does Borna disease virus infect humans?" *Nature Medicine*, vol. 1, no. 3, pp. 209–210, 1995.

[69] K. Lieb and P. Staeheli, "Borna disease virus—does it infect humans and cause psychiatric disorders?" *Journal of Clinical Virology*, vol. 21, no. 2, pp. 119–127, 2001.

[70] T. Briese, M. Hornig, and W. I. Lipkin, "Bornavirus immunopathogenesis in rodents: models for human neurological diseases," *Journal of NeuroVirology*, vol. 5, no. 6, pp. 604–612, 1999.

[71] M. V. Solbrig, G. F. Koob, J. N. Joyce, and W. I. Lipkin, "A neural substrate of hyperactivity in Borna disease: changes in brain dopamine receptors," *Virology*, vol. 222, no. 2, pp. 332–338, 1996.

[72] A. Hans, J. J. Bajramovic, S. Syan, et al., "Persistent, noncytolytic infection of neurons by Borna disease virus interferes with ERK 1/2 signaling and abrogates BDNF-induced synaptogenesis," *The FASEB Journal*, vol. 18, no. 7, pp. 863–865, 2004.

[73] M. Hornig, H. Weissenböck, N. Horscroft, and W. I. Lipkin, "An infection-based model of neurodevelopmental damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 21, pp. 12102–12107, 1999.

[74] M. V. Solbrig, G. F. Koob, J. H. Fallon, and W. I. Lipkin, "Tardive dyskinetic syndrome in rats infected with Borna disease virus," *Neurobiology of Disease*, vol. 1, no. 3, pp. 111–119, 1994.

[75] M. V. Solbrig, G. F. Koob, J. H. Fallon, S. Reid, and W. I. Lipkin, "Prefrontal cortex dysfunction in Borna disease virus

(BDV)-infected rats," *Biological Psychiatry*, vol. 40, no. 7, pp. 629–636, 1996.

[76] M. V. Solbrig, G. F. Koob, L. H. Parsons, et al., "Neurotrophic factor expression after CNS viral injury produces enhanced sensitivity to psychostimulants: potential mechanism for addiction vulnerability," *The Journal of Neuroscience*, vol. 20, no. 21, p. RC104, 2000.

[77] S. E. Hyman, "Can neuroscience be integrated into the DSM-V?" *Nature Reviews Neuroscience*, vol. 8, no. 9, pp. 725–732, 2007.

[78] Y. Lecrubier, "Refinement of diagnosis and disease classification in psychiatry," *European Archives of Psychiatry and Clinical Neuroscience*, vol. 258, no. 1, pp. 6–11, 2008.

[79] K. A. Phillips, S. Van Bebber, and A. M. Issa, "Diagnostics and biomarker development: priming the pipeline," *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 463–469, 2006.

[80] M. Leboyer, F. Bellivier, R. Jouvent, M. Nosten-Bertrand, J. Mallet, and D. Pauls, "Psychiatric genetics: search for phenotypes," *Trends in Neurosciences*, vol. 21, no. 3, pp. 102–105, 1998.

[81] I. I. Gottesman and J. Shields, "Genetic theorizing and schizophrenia," *British Journal of Psychiatry*, vol. 122, no. 566, pp. 15–30, 1973.

[82] T. D. Gould and I. I. Gottesman, "Psychiatric endophenotypes and the development of valid animal models," *Genes, Brain and Behavior*, vol. 5, no. 2, pp. 113–119, 2006.

[83] B. Arts, N. Jabben, L. Krabbendam, and J. Van Os, "Meta-analyses of cognitive functioning in euthymic bipolar patients and their first-degree relatives," *Psychological Medicine*, vol. 38, no. 6, pp. 771–785, 2008.

[84] B. Viswanath, Y. C. J. Reddy, K. J. Kumar, T. Kandavel, and C. R. Chandrashekar, "Cognitive endophenotypes in OCD: a study of unaffected siblings of probands with familial OCD," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 33, no. 4, pp. 610–615, 2009.

[85] H. S. Hoffman and J. R. Ison, "Reflex modification in the domain of startle: I. Some empirical findings and their implications for how the nervous system processes sensory input," *Psychological Review*, vol. 87, no. 2, pp. 175–189, 1980.

[86] D. L. Braff, C. Grillon, and M. A. Geyer, "Gating and habituation of the startle reflex in schizophrenic patients," *Archives of General Psychiatry*, vol. 49, no. 3, pp. 206–215, 1992.

[87] D. L. Braff, M. A. Geyer, and N. R. Swerdlow, "Human studies of prepulse inhibition of startle: Normal subjects, patient groups, and pharmacological studies," *Psychopharmacology*, vol. 156, no. 2-3, pp. 234–258, 2001.

[88] K. S. Cadenhead, N. R. Swerdlow, K. M. Shafer, M. Diaz, and D. L. Braff, "Modulation of the startle response and startle laterality in relatives of schizophrenic patients and in subjects with schizotypal personality disorder: evidence of inhibitory deficits," *American Journal of Psychiatry*, vol. 157, no. 10, pp. 1660–1668, 2000.

[89] D. L. Braff, R. Freedman, N. J. Schork, and I. I. Gottesman, "Deconstructing schizophrenia: an overview of the use of endophenotypes in order to understand a complex disorder," *Schizophrenia Bulletin*, vol. 33, no. 1, pp. 21–32, 2007.

[90] F. X. Castellanos and R. Tannock, "Neuroscience of attention-deficit/hyperactivity disorder: the search for endophenotypes," *Nature Reviews Neuroscience*, vol. 3, no. 8, pp. 617–628, 2002.

[91] R. H. Lenox, T. D. Gould, and H. K. Manji, "Endophenotypes in bipolar disorder," *American Journal of Medical Genetics*, vol. 114, no. 4, pp. 391–406, 2002.

[92] K. R. Merikangas, A. Chakravarti, S. O. Moldin, H. Araj, J. C. Blangero, and M. Burmeister, "Future of genetics of mood disorders research," *Biological Psychiatry*, vol. 52, no. 6, pp. 457–477, 2002.

[93] D. H. Skuse, "Endophenotypes and child psychiatry," *British Journal of Psychiatry*, vol. 178, pp. 395–396, 2001.

[94] C. E. Bearden, A. J. Jasinska, and N. B. Freimer, "Methodological issues in molecular genetic studies of mental disorders," *Annual Review of Clinical Psychology*, vol. 5, pp. 49–69, 2009.

[95] M. Rutter, "Biological implications of gene-environment interaction," *Journal of Abnormal Child Psychology*, vol. 36, no. 7, pp. 969–975, 2008.

[96] S. H. Fatemi, M. Araghi-Niknam, J. A. Laurence, J. M. Stary, S. Lee, and R. W. Sidwell, "Glial fibrillary acidic protein and glutamic acid decarboxylase 65 and 67 kDa proteins are increased in brains of neonatal BALB/c mice following viral infection in utero," *Schizophrenia Research*, vol. 69, no. 1, pp. 121–123, 2004.

[97] J. N. Crawley, "Behavioral phenotyping strategies for mutant mice," *Neuron*, vol. 57, no. 6, pp. 809–818, 2008.

[98] C. Belzung, "Measuring rodent exploratory behavior," in *Handbook of Molecular-Genetic Techniques for Brain and Behavior Research*, vol. 13 of *Techniques in the Behavioral and Neural Sciences*, pp. 738–749, Elsevier, Amsterdam, The Netherlands, 1999.

[99] S. H. Richter, J. P. Garner, and H. Würbel, "Environmental standardization: cure or cause of poor reproducibility in animal experiments?" *Nature Methods*, vol. 6, no. 4, pp. 257–261, 2009.

[100] J. L. Lathey and S. A. Spector, "Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages," *Journal of Virology*, vol. 65, no. 11, pp. 6371–6375, 1991.

[101] P. D. Markham, S. Z. Salahuddin, and K. Veren, "Hydrocortisone and some other hormones enhance the expression of HTLV-III," *International Journal of Cancer*, vol. 37, no. 1, pp. 67–72, 1986.

[102] V. Schuster, E. Dohrmann, and H. W. Kreth, "Epstein-Barr virus-associated lymphoproliferative syndromes: studies in two European families," *Cancer Detection and Prevention*, vol. 15, no. 1, pp. 65–67, 1991.

[103] C. W. Cotman, R. E. Brinton, A. Galaburda, B. McEwen, and D. M. Schneider, Eds., *The Neuro-Immuno-Endocrine Connection*, Raven Press, New York, NY, USA, 1987.

[104] F. Chaouloff, "Serotonin, stress and corticoids," *Journal of Psychopharmacology*, vol. 14, no. 2, pp. 139–151, 2000.

[105] D. M. Dietz, M. W. Vogel, S. A. Rubin, T. H. Moran, K. M. Carbone, and M. V. Pletnikov, "Developmental alterations in serotoninergic neurotransmission in Borna disease virus (BDV)-infected rats: a multidisciplinary analysis," *Journal of NeuroVirology*, vol. 10, no. 5, pp. 267–277, 2004.

[106] M. V. Pletnikov, S. A. Rubin, G. J. Schwartz, K. M. Carbone, and T. H. Moran, "Effects of neonatal rat Borna disease virus (BDV) infection on the postnatal development of the brain monoaminergic systems," *Developmental Brain Research*, vol. 119, no. 2, pp. 179–185, 2000.

[107] S. N. Goodman, "Toward evidence-based medical statistics. 1: the P value fallacy," *Annals of Internal Medicine*, vol. 130, no. 12, pp. 995–1004, 1999.

[108] S. N. Goodman, "Of p-values and bayes: p modest proposal," *Epidemiology*, vol. 12, no. 3, pp. 295–297, 2001.

[109] M. Cowles and C. Davis, "On the origins of the .05 level of statistical significance," *American Psychologist*, vol. 37, no. 5, pp. 553–558, 1982.

[110] I. Glegling, A. M. Hartmann, J. Genius, J. Benninghoff, H. J. Möller, and D. Rujescu, "Systems biology and complex neurobehavioral traits," *Pharmacopsychiatry*, vol. 41, no. 1, pp. S32–S36, 2008.

[111] H. H. H. Göring, A. G. Comuzzie, M. C. Mahaney, et al., "Discovery of expression QTLs using large-scale transcriptional profiling in human lymphocytes," *Nature Genetics*, vol. 39, no. 10, pp. 1208–1216, 2007.

[112] G. Pendyala, E. J. Want, W. Webb, G. Siuzdak, and H. S. Fox, "Biomarkers for neuroAIDS: the widening scope of metabolomics," *Journal of Neuroimmune Pharmacology*, vol. 2, no. 1, pp. 72–80, 2007.

[113] G. Pendyala, S. A. Trauger, E. Kalisiak, R. J. Ellis, G. Siuzdak, and H. S. Fox, "Cerebrospinal fluid proteomics reveals potential pathogenic changes in the brains of SIV-infected monkeys," *Journal of Proteome Research*, vol. 8, no. 5, pp. 2253–2260, 2009.

[114] B. L. Williams, K. Yaddanapudi, C. M. Kirk, A. Soman, M. Hornig, and W. I. Lipkin, "Metallothioneins and zinc dysregulation contribute to neurodevelopmental damage in a model of perinatal viral infection," *Brain Pathology*, vol. 16, no. 1, pp. 1–14, 2006.

[115] A. B. Niculescu 3rd and J. R. Kelsoe, "The human genome: genetic testing and animal models," *American Journal of Psychiatry*, vol. 158, no. 10, p. 1587, 2001.

[116] A. B. Niculescu, L. L. Lulow, C. A. Ogden, et al., "PhenoChipping of psychotic disorders: a novel approach for deconstructing and quantitating psychiatric phenotypes," *American Journal of Medical Genetics B*, vol. 141, no. 6, pp. 653–662, 2006.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 273573, 18 pages
doi:10.1155/2010/273573

*Review Article*

# *Chlamydophila pneumoniae* Infection and Its Role in Neurological Disorders

**Carlo Contini,[1] Silva Seraceni,[1] Rosario Cultrera,[1] Massimiliano Castellazzi,[2] Enrico Granieri,[2] and Enrico Fainardi[3]**

[1] *Section of Infectious Diseases, Department of Clinical and Experimental Medicine, National Institute of Neuroscience and Neuroscience Center, University of Ferrara, Via Fossato di Mortara, 23, 44100 Ferrara, Italy*
[2] *Section of Neurology, Department of Medical and Surgical Sciences of the Communication and Behaviour, Multiple Sclerosis Center, National Institute of Neuroscience and Neuroscience Center, University of Ferrara, Arcispedale S. Anna, Corso della Giovecca 203, 44100 Ferrara, Italy*
[3] *Neuroradiology Unit, Department of Neurosciences and Rehabilitation, Azienda Ospedaliera-Universitaria, National Institute of Neuroscience and Neuroscience Center, University of Ferrara, Arcispedale S. Anna, Corso della Giovecca 203, 44100 Ferrara, Italy*

Correspondence should be addressed to Carlo Contini, cnc@unife.it

Received 27 July 2009; Accepted 25 November 2009

Academic Editor: Guey Chuen Perng



*Chlamydophila pneumoniae* is an intracellular pathogen responsible for a number of different acute and chronic infections. The recent deepening of knowledge on the biology and the use of increasingly more sensitive and specific molecular techniques has allowed demonstration of *C. pneumoniae* in a large number of persons suffering from different diseases including cardiovascular (atherosclerosis and stroke) and central nervous system (CNS) disorders. Despite this, many important issues remain unanswered with regard to the role that *C. pneumoniae* may play in initiating atheroma or in the progression of the disease. A growing body of evidence concerns the involvement of this pathogen in chronic neurological disorders and particularly in Alzheimer's disease (AD) and Multiple Sclerosis (MS). Monocytes may traffic *C. pneumoniae* across the blood-brain-barrier, shed the organism in the CNS and induce neuroinflammation. The demonstration of *C. pneumoniae* by histopathological, molecular and culture techniques in the late-onset AD dementia has suggested a relationship between CNS infection with C. pneumoniae and the AD neuropathogenesis. In particular subsets of MS patients, *C. pneumoniae* could induce a chronic persistent brain infection acting as a cofactor in the development of the disease. The role of Chlamydia in the pathogenesis of mental or neurobehavioral disorders including schizophrenia and autism is uncertain and fragmentary and will require further confirmation.

## 1. Introduction and Background

*Chlamydiae* were taxonomically categorised into their own order *Chlamydiales*, with one family, *Chlamydiaceae*, and a single genus, *Chlamydia* which included four species: *C. trachomatis, C. pneumoniae, C. Psittaci*, and *C. pecorum*. Two of the species, *C. trachomatis* and *C. pneumoniae*, are common pathogens in humans, but the routes of transmission, susceptible populations, and clinical presentations differ markedly. The other species occur mainly in animals although *C. psittaci* may be also implicated in human respiratory diseases. In 1999, a new taxonomic classification was proposed, renaming *Chlamydia pneumoniae* as *Chlamydophila pneumoniae* [1]. However, the proposal to change the taxonomic nomenclature for the *Chlamyadiaceae* family has not been universally accepted and both names are currently in use by different authors.

*C. pneumoniae*, a common cause of human respiratory disease, was first isolated from the conjunctiva of a child in Taiwan in 1965 but it was not until the early 1980s that it was scientifically identified as a distinct *Chlamydia* species and was established as a major respiratory pathogen in 1983 when it was isolated from the throat of a college student at the University of Washington. Most likely, *C. pneumoniae* is primarily transmitted from human to human by the respiratory tract without any animal reservoir [2, 3] and infection spreads slowly. The incubation period is several weeks, which is longer than that for many other

respiratory pathogens [4]. The time span of infection spread in families is shorter, however, ranging from 5 to 18 days [5]. As other *Chlamydia* species, *C. pneumoniae* has a unique biphasic life cycle with two forms that are functionally and morphologically distinct that undergo an orderly alternation: the elementary body (EB), infectious and metabolically inactive responsible for attaching to the target host cell and promoting its entry and the reticulate body (RB), an abortive non-infectious and metabolically active intracellular form which replicates by binary fission and reorganizes into EBs then released by cell lysis. In general, it is likely that this aberrant developmental step leads to the persistence of viable but non cultivable *Chlamydiae* within infected cells over long periods. In cell culture conditions, the duration of the developmental cycle is between 2 and 3 days, depending on the strain, when bacteria have differentiated back to EBs and are released in the extracellular medium. In natural infections, the situation is more complicated, and the normal development of *Chlamydia* is easily disturbed. Living separated from the host cell cytoplasm within its Chlamydial inclusion (a nonlysosomal vacuole), *C. pneumoniae* is able to create an intracellular niche from where it promotes host cell survival or death, modulates regulatory host cell signalling pathways, and bypasses the host cell's defence mechanisms. Thus, *C. pneumoniae* can induce a persistent infection due to the inability of the host to completely eliminate the pathogen [6–8]. The failure by the guest to eradicate the disease involves the establishment of a state of chronic infection in which *C. pneumoniae* after internalization into mononuclear cells, enters into a state of quiescence with intermittent periods of replication and characterized by antigenic variation, production of Heat Shock Proteins (HsPs) and proinflammatory cytokines capable of evading host defences and trigger tissue damage [8]. Chronic infection and clinical persistence are closely related. In chronic infections a different pathway is taken. Under pressure from host defences the metabolic processes of the organism are diminished. *C. pneumoniae* in this state is called the Cryptic Body (CB). This chronic unresolved infection, which can last for several decades, can also initiate the malign process of autoimmunity. To a large extent, the form of the disease may depend on the host's genetic inheritance. This is why many of the chronic disease forms caused by infections with *C. pneumoniae* tend to have inherited characteristics. It is thought that the host immunity together with individual traditional risk factors, serological markers of *C. pneumoniae* infection and genetic susceptibility, may play an important role in controlling Chlamydial infections. A chronic *C. pneumoniae* infection increases the expression of its own 60 kDa heat shock proteins (HsP60), especially when they are persistently elevated. The host immune response to microbial HsP60 may gradually lead or, contribute to autoimmunity to human HsP60 and, consequently, to the development of some chronic diseases such as asthma [9] atherosclerosis or clinical manifestations of Coronary Hearth Disease (CHD) [4, 9–14]. Definitive pathogenesis and virulence investigations for the *Chlamydiae* have been hampered because methods for gene transfer have not yet been developed for these microorganisms. Despite this critical experimental limitation, a great deal of information has been generated on intracellular Chlamydial growth and development, and the effects of chlamydial infection on host-cell physiology [15]. Like most intracellular pathogens, *C. pneumoniae* interferes with the normal apoptotic signalling pathways of these cells, perhaps contributing to long-term persistence and chronic inflammation in central nervous system (CNS) system. However, it is not clear how this happens: under some circumstances these microorganisms induce apoptosis and/or necrosis, but under other circumstances they inhibit apoptosis [15, 16]. The circumstances that dictate whether the *Chlamydiae* inhibit or activate host-cell death reflect several important pathogenic considerations, including whether an acute or chronic infection is in progress and whether intracellular chlamydial growth is programmed to go through a productive infectious cycle or is stalled under non-productive growth conditions [16]. The intracellular growth cycle of the *Chlamydiae* is complex and several growth options are possible, depending on the host-cell type, the particular environmental conditions in the host cell and the nature of the tissue that is being affected. It is possible that apoptotic activity is controlled to some extent by the intracellular growth status of the *Chlamydiae*, which can be influenced by any or all of these considerations [16].

Data on the distribution of seroprevalence reveal that the prevalence of *C. pneumoniae* infection increases with age. Antibodies against *C. pneumoniae* begin to appear at school age but are rare in children under the age of 5, except in developing and tropical countries. Antibody prevalence increases rapidly at ages 5 to 14, reaches 50% at the age of 20, and continues to increase slowly to 70% to 80% at ages 60 to 70 [4]. This seems to suggest that most people are infected and re-infected for life. *C. pneumoniae* accounts for 6%–20% of community acquired pneumonia (CAP) in adults [3, 4], but participates in co-infection involving other bacterial agents in approximately 30% of adult cases of CAP [4]. Some studies have suggested a possible association of *C. pneumoniae* infection and acute exacerbations of asthma and chronic obstructive pulmonary diseases (COPD). In recent years, however, in addition to respiratory diseases, an increasing number of publications have been reported of detection of *C. pneumoniae* in chronic extra-respiratory diseases. In fact, the recent deepening of knowledge on the biology of *Chlamydia* and the use of increasingly more sensitive and specific molecular techniques, have allowed to demonstrate the presence of *C. pneumoniae* DNA in a large number of persons suffering from different diseases other than cardiovascular (atherosclerosis and stroke), such as osteoarthritis and CNS disorders. In this setting, the ability of *C. pneumoniae* to infect various human cells such as epithelial, endothelial, and smooth muscle cells as well as macrophages, monocytes, and lymphocytes, suggests a systemic dissemination following exposure to a respiratory infection. Certainly, the presence of *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMCs) strongly suggests that such dissemination can occur in a number of different tissues [17]. *C. pneumoniae* infection has been shown to promote the transmigration of monocytes through human brain endothelial cells, suggesting a mechanism by

which the organism may enter the CNS. This may account for the delivery of the organism to the CNS and result in chronic injury [18].

This review addresses the potential and the underlying mechanisms by which *C. pneumoniae* infections can play a role in different neurological diseases, examining the epidemiological and methodological findings among studies which will be highlighted. Suggestions for future studies and potential standardization of tools and protocols are proposed.

## 2. *C. pneumonia* Infection, Atherosclerosis, and Cerebrovascular Diseases

*2.1. C. pneumoniae and Atherosclerosis.* If large is the amount of data that support the role of *C. pneumoniae* in the atherosclerosis, equally important is the weight that *C. pneumoniae* has recently taken in the cerebrovascular diseases. The mechanism of atherosclerotic disease and thrombosis is not completely known. The first report of an association between *C. pneumoniae* infection and atherosclerosis was by Saikku et al. [2]. Individuals with immunoglobulin G antibody specific for *C. pneumoniae* were shown to be at an increased risk for myocardial infarction and CHD, as there was a statistically significant difference in the frequency of antibody in patients versus controls. Subsequently, many other reports concerning the seroepidemiological association between specific antibodies to *C. pneumoniae* and atherosclerosis at several arterial sites using retrospective, cross-sectional and prospective studies, have confirmed a link between *C. pneumoniae* and atherosclerosis [19]. Overall, these seroepidemiological studies have shown some limitations because different laboratories have measured different antibody classes, used different criteria for determining seropositivity or measured antibodies that were cross-reactive with other chlamydial species. Although most of the studies have used the micro-immunofluorescence test (MIF) for measuring antibodies, inter-laboratory variation in interpreting the results remains a problem [20, 21]. However, a number of experimental data support the involvement of this pathogen in the pathogenesis of atheroma [11–13]. First, *C. pneumoniae* gains access the vasculature during local inflammation in lower respiratory tract infection. Second, the infected alveolar macrophages transmigrate through the mucosal barrier and give the pathogen access to the lymphatic system, systemic circulation, and atheromas. Third, *C. pneumoniae* can infect a variety of cells commonly found in atheromas, including coronary artery endothelial cells, macrophages, and aortic smooth muscle cells. Fourth, *C. pneumoniae* may influence atheroma biology by modulating macrophage-lipoprotein interactions. In this setting, chlamydial lypopolysaccaride (LPS), a potent endotoxin constituent of outer membrane of Gram negative bacteria induces the release of cytokines promoting leucocyte adherence, leucocyte migration, and intimal inflammation. LPS has also been demonstrated to have a crucial role in lipid metabolism [22] and it is involved in mediating the process of "catching" LDL cholesterol by macrophages infected with *C. pneumoniae*, which is transformed into "foam cells," the "key cells" of newly formed atherosclerotic lesions [23]. Moreover, in vitro studies have shown that upregulated molecules by *C. pneumoniae*, including Ⓡ-2 and Ⓡ1-integrins, adhesion molecules (ICAM-1, VCAM-1), platelet derived growth factor (PDGF), tissue factor (TF), early growth response factor (EGR-1), appear to contribute to these events. Some of these, along with matrix metalloproteinases (MMPs) contribute to the destabilization of atheromasic plaque, and the formation of thrombus [12] and consecutively may lead to the arterial thromboembolic complications. The link between *C. pneumoniae* and coronary atherosclerosis is also substantiated by studies in which antibodies against members of the HsPs family were detected in individuals with persistent *C. pneumoniae* infections [9–11, 24, 25]. Several findings indicate in fact that HsPs, which are evolutionary very conservative and are present in both microbial and host cells, seem to be important in the development of autoimmunity. *C. pneumoniae* HsPs are expressed in abundance in atherosclerotic lesions and a persistent *Chlamydia* infection is accompanied by an increased production of HsP60. In particular, this protein, may represent a particular antigenic stimulus capable of eliciting strong humoral and cell-mediated immune responses with immunopathological sequelae of chronic chlamydial infections [9, 10, 12, 13, 26]. In this regard, *C. pneumoniae* Hsp60 has been demonstrated in patients with acute hemorrhagic evolution of the carotid plaque suggesting that *C. pneumoniae* might participate in the atherogenesis and to induce atherosclerosis complications by inflammatory pathways (activation of cytokines, endothelial factors and MMPs) [9]. At present, very little is known about genes predisposing the host to the persistent *C. pneumoniae* infection and its sequelae. HLA haplotype markers have been recently investigated in patients with coronary artery disease (CAD). In a recent study, a multiple logistic regression analysis showed HLA-B∗35 allele as the strongest risk gene for the combined serological markers of *C. pneumoniae*. Male sex and smoking further strengthened the association between HLA-B35 and markers of *C. pneumoniae* infection. Interestingly, HLA-B∗35 was also found to be associated with cardiovascular risk in Finnish patients [14].

*2.2. C. pneumoniae and Stroke.* The role of *C. pneumoniae* in the pathogenesis of ischemic stroke is still debated. Infection with *C. pneumoniae* may contribute to the risk of stroke by enhancing CAD, as addressed in several issues. Several published studies including the pilot case control "Northern Manhattan Stroke Study," focused on the correlation between the *C. pneumoniae* infection, represented by the elevated serum levels of specific anti-*C. pneumoniae* (IgA, IgG, and IgM) antibodies at MIF test and stroke occurrence [26–31], in different race-ethnic groups and after adjusting for conventional risk factors. The MIF method was used in most of the previously published papers, as considered as a reference standard in the *C. pneumoniae* serology. The elevated titers of anti-*C. pneumoniae* IgA and IgG were more prevalent in subjects with acute ischemic stroke than in controls [26, 29–32]. With immunohistochemical

staining, other investigators demonstrated the presence of *C. pneumoniae* in more than 70% of the endarterectomy specimens from stroke patients with severe carotid stenosis [33]. Other authors assessed anti-*Chlamydia* IgA and IgG seropositivity using an ELISA test and found an increased risk of stroke in young patients seropositive to *C. pneumoniae* in the IgA antibody class rather than in IgG class [34]. This has suggested the possibility that IgA antibodies, which last only 3 to 5 days in the circulation, are a diagnostic marker of persistent, chronic infection (single IgA MIF titer $\geq 1:16$), whereas IgG antibodies, which are produced for 3 to 5 years, are a marker of older, inactive infection [14]. The association between IgA titers and risk of ischemic stroke was also stratified according to ischemic stroke subtype. In contrast with the less consistent evidence of an association of IgA titers and cardioembolic and cryptogenic stroke, there was a trend toward an association of IgA titers with large vessel atherosclerotic and lacunar stroke, strongly supporting evidence that *C. pneumoniae* contributes to atherosclerosis [30]. However, according to a previous consensus statement [35] and other authors [36, 37], there is not yet agreement that IgA titers are indicative of chronic, persistent infection. First, the half-life of specific *C. pneumoniae* is only a few days. Second, measurement of IgA antibodies may be complicated by cross-reacting *C. pneumoniae* IgG and rheumatoid factor or with antibodies to other chlamydial species and potentially other microorganisms. Third, MIF, the serologic "golden standard" which may have been overestimated in the past, is not standardized and there are interlaboratory variations in the performance of this test [38]. Finally, the hypothesis that *C. pneumoniae* infection, as indicated by elevated IgA and IgG antibody titers, may be not associated with an increased risk of ischemic stroke. This, however, may differ according to subtype of ischemic stroke, cut-off value of antibody titers, and gender. Anti-*C. pneumoniae* antibodies were also evaluated in HIV infected individuals with CAD. One study showed that both the IgA and IgG levels did not differ significantly and no subjects were positive for IgM, suggesting that the damage to the carotid wall in HIV-1 patients was not due to *C. pneumoniae* [39]. Other studies by contrast, found that *C. pneumoniae* represents a further risk factor for cardiovascular disease in HIV-positive patients with both low CD4 cell count and high HIV load [40] and that the coexistence of hypertriglyceridemia and *C. pneumoniae* infection significantly increases the risk of atherosclerosis up to three times [41].

A number of studies addressed the question of whether molecular tools and in particular PCR can be used to detect chlamydial DNA in cerebrovascular atherosclerotic lesions. The results provided from a consistent number of atherosclerotic lesion and blood specimens from more than 1500 patients analysed by different in-house PCRs, were found extremely variable. Being PCR not standardized, this technique has a tendency to produce false positive results. In this setting, there is the need for standardization of PCR methods and for assessing their sensitivity, specificity, and predictive values. In general, there is not consensus on how nested PCR (n-PCR) technology can be controlled [42]. PCRs have been shown to be predictive of CAD, when used for detection of *C. pneumoniae* DNA in PBMC, suggesting that the detection of bacterial DNA in PBMC may be a valid surrogate marker to identify individual risk for endovascular chlamydial infection [43]. Recently, *C. pneumoniae* DNA and chlamydial LPS were measured during 12 months in 97 patients with acute CAD. *C. pneumoniae* DNA was detected in PBMC from 8 (8.2%) patients at the initial hospitalization during acute CAD and in 9 (10.6%) patients at 3 months. *C. pneumoniae* DNA declined in stable state and in the recovery period. These findings suggest a role of the bacterium in the acute phase of CAD [44]. Attempts to eradicate *C. pneumoniae* in patients with cardiovascular diseases including the "Azytromycin and Coronary Event Study" trial, have all failed [45–47]. As shown in previous studies the persistent state is completely refractory to antibiotic treatment [45] and first-choice antichlamydial drugs may even induce chlamydial persistence under certain conditions [46]. In the absence of a functional treatment strategy, the hypothesis of a chlamydial contribution to atherogenetic processes can thus neither be proved nor disproved by eradication studies, and a better understanding of chlamydial pathobiology in order to target specific chlamydial antigens is needed before implementing clinical studies with new effective antibiotic regimens.

In summary a causative role of *C. pneumoniae* infection in cardiovascular disease has not yet been firmly established. Despite the considerable laboratory and clinical research that has been done on the role of *C. pneumoniae* in the progression of atherosclerosis, several important questions remain unanswered. Most importantly, it is not known whether the *C. pneumoniae* bacterium is an innocent passenger aboard atheromas or whether it is actively involved in the initiation or progression of atherosclerotic disease. To answer this question, well-planned studies are needed to further characterize the molecular mechanisms that link *C. pneumoniae* to vascular disease. In particular, HsP60 needs to be explored further as a potential culprit and therapeutic target. The early results from antichlamydial intervention studies in humans are partially consistent with a causative role of *C. pneumoniae* in the disease process.

## 3. *C. pneumoniae*, Neurodegenerative, and Demyelinating Disorders

The ability of the *C. pneumoniae* to persist in monocytes and macrophages in tissues for long periods, to circumvent the mechanisms of bactericidal and oxidative stress, to activate the endothelial cells with production of adhesion molecules and cytokine overproduction, has suggested that it may participate in the development or progression of certain acute and chronic inflammatory diseases of the CNS. In recent years, in fact, a growing body of evidence concerning the involvement of *C. pneumoniae* in neurological diseases has been gradually increasing. This was supported in particular by the detection of genomic material of the microrganism into the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS), Alzheimer's disease (AD), meningoencephalitis and neurobehavioral disorders [4, 6–8, 18].

*3.1. Alzheimer's Disease.* Alzheimer's disease (AD) is one of the most severe dementing illnesses that increases with the increasing age of the population. The disease is associated with atrophy/death of neurons in particular regions of the brain and occurs in two general forms: an early-onset form that is primarily genetically determined, and a far more common late-onset AD (LOAD), which is a non-familial, progressive neurodegenerative disease that is now the most common and severe form of dementia in the elderly. The defining neuropathology of both familial and sporadic AD includes the neuritic senile plaque (NSPs), consisting primarily of amyloid beta (A$\beta$) protein, and neurofibrillary tangles (NFTs), the major component being modified *tau* protein, that affect nerve synapses and nerve-nerve cell communication. Genetic, biochemical, and immunological analyses have in part provided a relatively detailed knowledge of these entities [48, 55]. The disease usually manifests initially as a gradual loss of short-term memory and later progresses to major cognitive dysfunction. The latter can take the form of various behavioural disorders, loss of orientation, difficulties with language, and impairment of daily living [56]. Estimates of crude incidence of AD range from 7.03 to 23.8 per 1000 person years [57, 58]. The range is likely attributable to different study populations and case investigating methods. The incidence of AD increases with age for both genders, but there is definite indication if there are gender differences. AD seems to be more common among women with approximately a third higher incidence and prevalence among females compared to males [59–61]. Using age-specific incidence rates one projection study in the U.S. predicted that incident cases would increase from the 360,000 cases in 1997 to 1.14 million in 2047 [61]. Despite the AD's discovery by Alois Alzheimer in 1907, the cause of this pathology and neurodegeneration is not unknown. Infections of the CNS have been shown to stimulate inflammatory responses that may result in neurodegeneration [62]. Several groups have investigated an association between various infectious agents and AD, but none of these has been accepted as either etiological for disease development, or worsening of neuropathology. Interesting perspectives came from one study which identified herpes simplex virus type 1 (HSV-1) infection as a risk factor for development of AD in subjects expressing APOE $\varepsilon$4 allele [63, 64]. Virus including measles virus, adenovirus, lentiviruses, and several other others were initially considered but discarded after [65, 66]. Bacterial pathogens including *C. trachomatis, Coxiella burnettii, Mycoplama* spp and spirochetes [67, 68] have also been investigated and dismissed in relation to involvement in AD neuropathogenesis. Prions have been also taken into consideration but then excluded [69]. The first paper that reported an association between *C. pneumoniae* infection and LOAD was from Balin et al. [48]. He found that 90% of AD brains were positive for *C. pneumoniae* as assessed by highly sensitive and specific PCR and the organism was detected in various sections of brain (hippocampus, cerebellum, temporal cortex, and prefrontal cortex) that exhibited AD pathology of more or less variable intensity [55]. Electron microscopic (EM) results revealed *C. pneumoniae* like particles containing EBs and RBs in the brain tissue and immunohistochemistry analysis indicated strong labelling in the sections of the brain most affected by AD among the cases, while no labelling in the controls. Moreover, a part the detection and visualization of *C. pneumoniae* within the cells of CNS that were associated with plaques and tangles, RNA transcripts of *C. pneumoniae* indicating metabolically active organisms were demonstrated by RT-PCR in frozen tissue samples and organisms were then isolated from the tissue and propagated in cell cultures. In that report, a strong association of APOE-$\varepsilon$4 genotype and *C. pneumoniae* infection was found in 58% (11/19) patients with AD suggesting, as shown in reactive arthritis, that the APOE-$\varepsilon$4 gene may promote some aspects of *C. pneumoniae* pathobiology in AD [48]. The Balin study report received a great deal of public and scientific attention and attempts to replicate the finding were conducted in mother reputable laboratories throughout the world. Two independent investigators (Ossewaarde et al., 2000 and Mahony et al., 2000, unpublished data) found *C. pneumoniae* in brains of AD patients with PCR and immunohistochemistry, validating Balin's results. However, conflicting results were found in subsequent studies by different authors using the same procedures but different protocols in paraffin-embedded brain tissues [49–53] attempting to revalidate the previous findings (Table 1). These studies have yielded contradictory results likely due to differences in diagnostic criteria and diagnostic tools. Demographic differences between the patient groups, such as geographic location, season of death and institutional history might also account for these opposing results. AD patients included in the Balin study, might have been recently exposed to *C. pneumoniae*, perhaps in an institutional setting, and therefore would have been at high risk of systemic spread from the respiratory tract to sites within the CNS, where advanced AD pathology already existed [70]. In a extension of these findings, Gerard, two years later, demonstrated *C. pneumoniae* in 80% of AD samples and 11.1% of the controls using primer targeting two *Chlamydia* (1046, 0695) genes. The AD cases (mean age 79.3 years) and controls (65.9 years) were age-and sex-matched as closely as possible, but the controls were younger and 22.7% were males [54]. The organism was again viable within the AD brain as assessed by culture of the organism from brain samples; moreover, RT-PCR analyses identified primary rRNA gene transcripts from *C. pneumoniae* indicating metabolic activity of the organism in those tissues. Interestingly, immunohistochemical analyses have also shown that astrocytes, microglia, and neurons all served as host cells for *C. pneumoniae* in the AD brain, and that infected cells were found in close proximity to both NSPs and NFTs in the AD brain (Figure 1). Recently, cultured astrocytes and microglia have also shown that *C. pneumoniae* displays an active, not a persistent, growth phenotype indicating probable concomitant destruction by lysis of some portion of host cells at the termination of that cycle [71]. In the years immediately after the study of Balin, some experimental discoveries have provided insights about the pathogenetic mechanisms of AD. First, a relationship exists between possession of the APOE-$\varepsilon$4 allele and the pathobiology of *C. Pneumonia* [72] and that

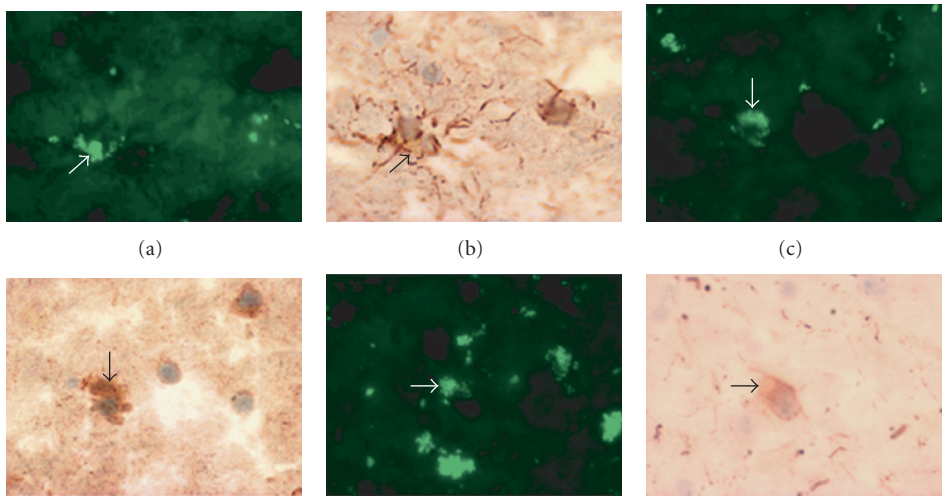



Figure 1: Representative images of double immunolabelling studies to demonstrate the infection of astrocytes, microglia, and neurons with Chlamydia pneumoniae in the AD brain. Chlamydia pneumoniae-infected cells were identified in all cases using the FITC-labelled monoclonal antibody targeting the *Chlamydia* LPS (Pathfinder TM; a, c, e). Astrocytes (b) and microglia (d) were identified by immunostaining using monoclonal antibodies targeting GFAP and iNOS, respectively. Neurons were identified by immunostaining with a monoclonal antibody targeting neuron-specific microtubule-associated protein (f). Images in all panels were obtained using a objective. In all panels, arrows indicate cells labelling with the Pathfinder TM and surface marker-specific monoclonal antibodies. (Reproduced from [54] with courtesy of Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI, USA, and with permission of *FEMS Med Lett*).

Table 1: Studies demonstrating evidence or absence of *C. pneumoniae* in brain autoptic specimens from patients with Alzheimer disease.

| Author, year | No. of autoptic AD brain specimens examined | *C. pneumoniae* DNA detection and confirmation methods | Results of ^PCR in AD brain Positive % | Results of PCR in control brain Positive % | Study of APOEe 4 gene expression |
|---|---|---|---|---|---|
| Balin et al. 1998 [48] | 19 | §PCR, ≠ RT-PCR, ^^IHM, *EM, Culture | 1790 | 15,2 | Yes |
| Nochlin et al. 1999 [49] | 13 | †PCR, IHM | 0 - | 0 - | Not done |
| Gieffers et al. 2000 [50] | 20 | °PCR, IHM | 0 - | 0 - | Not done |
| Ring and Lyons 2000 [51] | 15 | °°PCR, Culture | 0 - | 0 - | Not done |
| Taylor et al. 2002 [52] | 10 | •PCR, IHM | 0 - | 0 - | Not done |
| Wozniak et al. 2003 [53] | 4 | °°°PCR | 0 - | 0 - | Not done |
| Gérard et al. 2006 [54] | 27 | ‡PCR, RT-PCR, IHM, EM, Culture | 2580 | 311 | Yes |

^PCR, polymerase chain reaction; ≠ RT-PCR, Reverse transcriptase PCR; ^^IHM, immunoistochemistry; *EM, electron microscopy; §primers targeting the 16S ribosomal RNA (rRNA) gene (Gaydos et al., J Clin Microbiol 1992; 30: 796–800) and the chlamydial major outer membrane protein (MOMP) gene (*ompA*) (Perez-Melgosa et al., Infect Imm 1991; 59: 2195–9); †PCR with seminested primer amplifying *C. pneumoniae*-specific DNA sequences of 437 bp (Kuo et al, J Infect Dis 1993; 167: 841-9); °nested PCR (Maass et al., Atherosclerosis 1998; 140: S25–30); °°Primers targeting the MOMP gene (Perez-Melgosa et al., 1991; Infect Imm 1991; 59: 2195–9) •PCR using the 76 kDa protein gene [47], the *rpoB* gene encoding the RNA polymerase beta subunit (Ouchi et al., J Med Microbiol 1998; 47: 907–13), and the pan-*Chlamydia* primers targeting the 16S ribosomal RNA gene in *Chlamydia* and *Chlamydia*-like organisms (Ossewaarde et al., Microbiology 1999; 145: 411–17); °°°PCR with primer targeting the 16SrRNA gene (J Clin Microbiol 1992; 30: 796–800); ‡PCR-multiple assays targeting the Cpn1046 gene (aromatic amino acid hydroxylase) and Cpn0695 (MOMP)[54].

the *C. pneumoniae* load in the AD brain varies with APOE genotype [73]. Second, infection of human microvascular endothelial cells co-cultured with *C. pneumoniae* elicits increased expression of proteins relevant to access for the organism to CNS including N-cadherin and b-catenin [74]. Third, the expression of occludin, a protein associated with tight junctions, is attenuated in the *C. pneumoniae*-infected cells. Fourth, infection with *C. pneumoniae* through the olfactory pathways of nontransgenic young female BALB/c mice which usually do not develop AD, has shown to

promote the production of extracellular amyloidlike plaques (A$\beta$1-42) in the mouse's brain thus providing a unique model for the study of sporadic AD and suggesting that this model could be a primary trigger for AD pathology following a noninvasive route inoculation [75]. Since *C. pneumoniae* is harboured in the respiratory tract and has a predilection for infecting epithelial cells, the olfactory neuroepithelia in the nasal passages are a likely target for infection. Following entry into these epithelia, potential damage and/or cell death may occur in the main olfactory bulb and olfactory cortex, thereby setting the stage for further retrograde neuronal damage [55].

Because chlamydial chronic infections are characterized by the inaccessibility of the "chlamydial persistent state" to conventional antichlamydial agents, there are few clinical trials that have determined the effectiveness of antibiotic therapy against *C. pneumoniae* in AD. A first randomized, placebo controlled, multicentre clinical trial performed to determine whether a 3-month course of doxycycline and rifampin could reduce the decline of cognitive function in patients with AD showed significantly less cognitive decline at 6 months and less dysfunctional behaviour at 3 months, in the antibiotic group than in controls [76]. Although these observations do not demonstrate a causal relationship between CNS infection with *C. pneumoniae* in terms of eradication of chronic *C. pneumoniae* infection and the AD neuropathogenesis, they do open the way to further investigations. In this regard, animal modelling will be required to define in detail how chlamydial infection might result in AD-related pathological change in the CNS and to provide a better understanding of infection parameters. In vitro and mouse model studies have demonstrated that metal protein attenuating compounds (MPACs) promote the solubilisation and clearance of extracellular senile plaques comprised of beta-amyloid. The role of the antiprotozoal metal chelator clioquinol in AD, which has been reported to reduce beta-amyloid plaques, presumably by chelation associated with copper and zinc, is currently in clinical trials as potential for treatment of AD [77, 78]. The scientific knowledge surrounding Alzheimer's disease and infection by *C. pneumoniae* is still growing. Standardization of diagnostic techniques would certainly allow for better comparability of studies. However, other systemic infections as potential contributors to the pathogenesis of AD should be considered.

*3.2. C. pneumoniae and AIDS Dementia.* Many authors have explored the possibility that *C. pneumoniae* was involved in other neurodegenerative disorders other than Alzheimer's disease. The existing data are however few and not significant. Among those characterized by dementia, one study performed by our group explored the possible link between AIDS-dementia complex (ADC) and *C. pneumonia* [79]. ADC is an HIV-derived neuropathological disorder characterised by infection of macrophages and microglia cells and release of proinflammatory cytokines into the parenchyma [80]. In this report, *C. pneumoniae* was identified in the CNS by PCR for *C. pneumoniae* MOMP and 16S rRNA gene in 4 (17.4%) out of 23 HIV-infected patients diagnosed as ADC Stage 3 according to scheme for AIDS dementia complex and confirmed by autopsy. Sequence analysis revealed significant homologies with *C. pneumoniae* compared to *C. trachomatis* and *C. psittaci*. Moreover, high mean levels of CSF specific anti-*C. pneumoniae* antibodies and *C. pneumoniae* antibody specific index values significantly elevated were also found by ELISA in these patients. These findings suggest that although the low rate of isolation is not representative of the frequency with which *C. pneumoniae* is involved in the causation of CNS injury, in the late-stage HIV infection, an increase in "trafficking" of monocytes containing *C. pneumoniae* to the brain may carry this organism in the sites which are the major reservoirs of productive HIV replication and contribute to neuronal damage in HIV-infected patients [79]. Moreover, the possibility that may exist a patient's subgroup in whom this organism is not, as for atherosclerosis and other Chlamydial diseases, an "innocent bystander," but may survive and replicate in CNS macrophages cannot be excluded [8].

*3.3. C. pneumoniae and Multiple Sclerosis.* MS disease is a presumed autoimmune chronic inflammatory disease of the CNS of unknown aetiology triggered by an environmental factor in susceptible individuals. It generally affects 1 to 1.8 per 1,000 individuals and kills more than 3000 people each year, with a further estimated annual morbidity cost of over $ 2.5 billion. In the United States, the prevalence of the diseases is 250,000 to 350,000 cases annually [97]. The pathological hallmark of multiple sclerosis (MS) is the demyelinating plaque that represents an area of demyelination and gliosis around blood vessels [98]. Acute lesions show perivascular lymphocytes and plasma cells along with the infiltration of macrophages and phagocytosis of myelin membranes. The continuous breakdown and regeneration of myelin has been demonstrated within the progressive MS plaque [99]. Toll-like receptors (TLR) are intimately involved in several neurodegenerative and demyelinating disorders including MS as demonstrated with the finding of a marked increase in TLR expression in MS lesions. PCR studies have shown that microglial cells from MS patients express TLRs 1–8 [100]. Moreover, while healthy white matter from MS patients does not contain TLRs, active lesions are associated with high expression of TLR3 and TLR4 on microglia and astrocytes. In contrast, late active lesions also contain astrocytes bearing surface TLR3 and TLR4 [100]. This suggests that early lesions are characterized by microglia infiltration, while astrocytes are also active in later MS lesions. However, the precise role of TLR3 and TLR4 activation in these lesions is yet unknown. TLRs have been shown to recognize highly conserved regions in various microorganisms (Pathogen-Associated Molecular Patterns) including *C. pneumoniae* and thus stimulate a potent inflammatory response contributing to the clearance of the pathogen [101]. Unpublished our findings have detected the major expression of mRNA TLR-2 and TLR-4 in peripheral blood but not in CSF from SM patients with RR forms, indicating that their combined activity might be crucial to modulate and activate the cellular-mediated immune response during chronic infections by *C. pneumoniae* [102]. Based on epidemiological observations, it

has been proposed that exposure to an environmental factor, such as an infectious agent, in combination with genetic predisposition could be implicated in MS pathogenesis [103]. The risk of MS is enhanced by the presence of specific genes on chromosome 6 in the area of MHC, Human Leukocyte Antigens (HLA) in humans. In particular, HLA-DR and HLA-DQ genes, which are involved in antigen presentation, are strongly associated to the development of the disease. However, although the risk of the disease is higher in monozygotic than in dizygotic twins (about 30% and 5%, resp.), the low concordance rate obtained in identical twins suggests that non-genetic factors can contribute to MS aetiology. In this setting, the aetiopathogenesis of MS disease is complex and still debated. So far, about 20 microorganisms including viruses have been associated with this disease [104]. The screening techniques in these studies varied from serology to PCR and quality and numbers of controls examined varied widely. The latest pathogen to be associated with MS is *C. pneumoniae* [105–110]. Sriram et al. reported the first evidence suggesting the potential role for *C. pneumoniae* as a candidate in MS pathogenesis [106]. One year later, a larger study from the same group strongly confirmed that CSF demonstration of *C. pneumoniae* was more frequent in MS patients than in control patients with other neurological disorders (OND) [107]. In particular, *C. pneumoniae* culture isolation was obtained in 24/37 (65%) MS and in 3/27 (11%) OND patients, CSF single polymerase chain reaction (PCR) for major outer-membrane protein (MOMP) was positive in 36/37 (97%) MS and in 5/27 (18%) OND patients, whereas CSF anti-*C. pneumoniae* IgG were detected by enzyme linked immunoadsorbent assay (ELISA) in 32/37 (86%) MS and in 0/27 (0%) OND patients. After this innovative publication, a number of studies have suggested that *C. pneumoniae* infection may be associated with MS, while other studies have found no association [108, 109]. During recent years, there have been many evidences of a possible role of *C. pneumoniae* involvement in MS disease supported in part by seroepidemiological, cultural, molecular, immunological and therapeutic studies. However, it is also true that there are not many studies that argue for a role of organism in MS. First, while some reports have documented that *C. pneumoniae* seropositivity was related to the risk of MS progressive forms (SP and PP), but only moderately linked to the risk of developing MS [110], others have not found association between serum titers of anti-*C. pneumoniae* antibodies and the risk for MS or, by contrast, a higher risk to develop MS in a subgroup of older patients after than before disease onset [111]. Second, the organism was found in course of MS relapses in the throat together with a rising serology [112]. Third, relapses of MS have long been noted to follow respiratory infections, including sore throat, or pneumonia with a clinical pattern typical of respiratory infection caused by *C. pneumoniae*. The isolation of the pathogen, as assessed by culture assay in CSF and brain tissue failed repeatedly in MS patients [113–115] or was positive only in a small proportion of MS patients [81, 116]. Dong-Si et al. have noted gene transcription of messenger RNA by *C. pneumoniae* in CSF from MS patients suggesting active infection by this pathogen [91]. Recently, active transcription of DNA from the organism has been found in a persistent and metabolically active state in cultured CSF and PBMCs from MS patients, but not in controls [95]. Other investigators were able to culture and detect *C. pneumoniae* in buffy coat samples from a healthy blood donor population [117] demonstrating a *Chlamydia* carriage rate of 24.6%, within the WBC of the peripheral circulation. Because of the difficulties of isolating *C. pneumoniae* cultures, nucleic acid amplification methods such as PCR-based assays have become the method of choice for detection of this microorganism. However, PCR procedures often differ in several aspects which can affect sensitivity, reproducibility, and specificity when applied to direct testing of clinical specimens [86, 118, 119]. In this context, collaborative studies involving different laboratories in which the presence of *C. pneumoniae* was evaluated in blinded CSF samples, further underlined the lack of an accepted standardized PCR protocol [120, 121]. A number of PCR studies did not provide evidence of detection of *C. pneumoniae* DNA in CSF of MS patients. Most of these studies were performed using single or nested (n) PCR targeting either MOMP or 16S ribosomal (rRNA) chlamydial genes [11, 34, 114, 116, 122–124]. By contrast, a substantial body of work from around the world has provided clear evidence of the involvement of *C. pneumoniae* in MS. In this setting, a consistent number of studies did found PCR positive results with DNA or mRNA positive rates varying from 2.9% to 69% [81–91]; [94–96]; [117–126] as listed in Table 2. Some reports also demonstrated the more frequency of *C. pneumoniae* DNA in CSF of MS patients with Gd enhancing lesions on MRI scans [87, 89]. Moreover, CSF detection of heat-shock protein-60 messenger RNA (Hsp-60 mRNA) and 16S rRNA by Reverse-Transcriptase PCR (RT-PCR) was more frequent in MS patients than in controls signifying the presence of a high rate of gene transcription and, therefore, more active metabolism of *C. pneumoniae* in MS [91]. In 2004, our group developed a novel amplification program for MOMP gene by employing a "touchdown" technique and analyzing CSF samples from patients with MS, other inflammatory neurological disorders (OIND) and noninflammatory neurological disorders (NIND) and employed three gene targets (MOMP, 16S rRNA and HsP-70) in parallel to achieve a major sensitivity and specificity [92]. A PCR positivity for MOMP and 16S rRNA in CSF was present in a small proportion of MS (37%), OIND (28%) and NIND (37%) patients, without any differences between MS and controls. Furthermore, a PCR positivity for MOMP and 16S rRNA in CSF was more frequent in relapsing-remitting (RR) MS than in MS progressive forms (SP and in PP MS) as well as in clinically and magnetic resonance imaging (MRI) active than in clinically and MRI stable MS, whereas a CSF PCR positivity for HsP-70 was observed in only three active RR MS patients. Thus, it cannot be excluded that, in a particular subgroup of RR active MS patients, *C. pneumoniae* may enter into brain early in the course of the disease via transendothelial migration across the blood/brain barrier of activated infected blood-borne monocytes, resulting in ongoing inflammatory immune activation that takes place within the CNS. Alternatively, the presence of elevated rates

Table 2: Molecular protocols employed for detection of *C. pneumoniae* in clinical specimens from MS patients.

| MS patients (positive/total) | Controls (positive/total) | PCR assay | References |
|---|---|---|---|
| 7/30 (23%) | 0/56 (0%) | n-PCR† | Layh-Schmitt et al. 2000 [81] |
| 9/17 (52%) | 13/15 (86%) | PCR† | Li et al. 2000 [82] |
| 2/8 (25%) | | not reported† | Treib et al. 2000 [83] |
| 12/58 (21%) | 20/47 (43%) | n-PCR† | Gieffers et al. 2001 [84] |
| 3/32 (9%) | 0/30 (0%) | PCR† | Sotgiu et al. 2001 [85] |
| 11/16 (69%) | | PCR† | Ikejima et al. 2001 [86] |
| 9/28 (32%) | 2/15 (13%) | PCR† | Hao et al. 2002 [87] |
| 2/70 (2.9%) | 0/30 (0%) | n-PCR† | Chatzipanagiotou et al. 2003 [88] |
| 23/107 (21%) | 2/77 (3%) | Touchdown n-PCR† | Grimaldi et al. 2003 [89] |
| 2/25 (8%) | 3/28 (11%) | n-PCR and semi-n-PCR† | Rostasy et al. 2003 [90] |
| 42/84 (50%) | 25/89 (28%) | Touchdown n-PCR, RT-PCR† | Dong-Si et al. 2004 [91] |
| 26/71 (36.6%) | 24/72 (33.3%) | Touchdown n-PCR† | Contini et al. 2004 [92] |
| 12/20 (60%) | 1/12 (8%) | Touchdown n-PCR† | Sriram et al. 2005 [93] |
| 2/112 (2%) | 0/110 (0%) | Real-time PCR‡ | Sessa et al. 2007 [94] |
| 7–9/14 (50–64.3%)† | 0-1/19 (0–5.2%)† | Touchdown n-PCR, | Contini et al. 2008 [95] |
| 5–7/14 (35.7–50%)‡ | 0–2/19 (0–10.5 %)‡ | RT-PCR†‡, | |
| 64/80 (80%) | 5/57 (9%) | PCR-EIA, Touchdown n-PCR, Real Time-PCR† | Tang et al. 2009 [96] |

n-PCR: nested PCR; RT-PCR: reverse transcriptase PCR; PCR-EIA: PCR enzyme immunoassay; †, CFS: cerebrospinal fluid; ‡, PBMC: peripheral blood mononuclear cells.

of *C. pneumoniae* DNA in CSF in this subset of MS patients could merely reflect the selective infiltration of monocytes which traffic into the brain after activation, thus suggesting a role for *C. pneumoniae* only as a silent passenger. In attempting to recover *C. pneumoniae* from cultured CSF and PBMC compartments with a PCR targeting multiple genes, a positivity for *C. pneumoniae* DNA and mRNA was recently detected in 64% of cocultured CSF and PBMCs of RR MS patients with evidence of disease activity, whereas only 3 controls were positive for Chlamydial DNA, suggesting that *C. pneumoniae* may occur in a persistent and metabolically active state at both peripheral and intrathecal levels in MS, but not in controls [95]. In this study the parallel molecular analysis of multiple Chlamydial target genes after co-culture of fresh CSF and PBMC specimens, has shown to enhance the sensitivity and specificity of molecular tools. Of note, as *C. pneumoniae* DNA was found in PBMCs which are able to cross the blood-brain barrier, these cells could be the source of intrathecally compartmentalized *C. pneumoniae* that, in turn, may induce a chronic persistent brain infection acting as a cofactor in the development of the disease. More recently, in a comparative study aimed to evaluate novel procedures for the detection of *C. pneumoniae* DNA in CSF, the qualitative colorimetric microtiter plate-based PCR-enzyme-immunoassay (PCR-EIA) has shown to be more sensitive than a real-time quantitative PCR assay (TaqMan) and possessed a sensitivity that was equal to the nested-PCR [96]. In order to support the theory of an association between *C. pneumoniae* and MS, a number of studies did evaluate the presence of intrathecal IgG in the form of oligoclonal bands (OCB) in the CSF of MS patients. Their presence, as for other bacterial, viral, fungal, and parasitic diseases, would be of great evidence for an infectious cause of MS [127] and may reflect an antigen-driven immune response to infectious agents [128]. However, OCB are also detected other than in MS in 10% of patients with other inflammatory diseases of the CNS. In this regard, studies aimed to determine the CSF levels of anti-*C. pneumoniae* IgG in MS patients did result extremely variable (varying from 0% to 20%) producing any or scarce differences between MS and controls [81, 85, 87, 90, 122, 123, 126, 129, 130]. Recently, we found that an intrathecal synthesis of anti-*C. pneumoniae* IgG as evaluated by antibody specific index (ASI) was more frequent in MS (16.9%) and in OIND (21.6%) than in NIND (1.9%) patients and in patients with MS progressive forms (SP and PP MS) than in RR MS patients [131]. Moreover, among the patients with intrathecally produced anti-*C. pneumoniae* IgG, CSF *C. pneumoniae*-specific high-affinity antibodies, were demonstrated to be more frequent in a subset of patients with MS progressive forms (SP or

PP MS), than in OIND patients, and absent in RR MS and NIND patients. To further examine a possible relationship between *C. pneumoniae* infection and MS, Sriram published a study that examined autoptic samples of brain tissue and CSF using immunohistochemical staining with anti-*C. pneumoniae* monoclonal antibodies other than molecular and ultrastructural methods [93]. These techniques provided evidence of the presence of *C. pneumoniae* more commonly in MS patients (90%, 62%, and 55%, resp.) than in control patients. Using electron microscopy the authors first demonstrated the presence of immunogold-labeled objects of the morphology and size and of chlamydial EBs in the ependymal surfaces and periventricular regions in the CSF of four out of ten (40%) patients with MS but not in the CSF of control patients. Collectively taken, although MS patients were found to be more likely to have detectable levels of *C. pneumoniae* DNA in their CSF and intrathecally synthesized immunoglobulins, compared with patients with had neurological diseases, the overall findings examined in a review through 26 studies that considered 1332 MS patients and 1464 controls using random-effects methods and random-effects meta-regressions, adjusted for the confounding effect of gender differences, were insufficient to establish an etiologic relation between *C. pneumoniae* and MS [132].

The treatment directed against the inflammatory process is only partially efficacious on the MS disease course. In relapsing-remitting MS, such therapy slows the progression of disability, but in primary-progressive MS the same therapy has demonstrated to have little or no effect on the progression of disability. On the other hand, reports regarding the antimicrobial treatment of MS have provided conflicting results. In one trial the antibiotic minocycline resulted in a reduction in the number of gadolinium-enhancing MRI-detected lesions [133]. Another study showed that anti-chlamydial treatment reduced brain atrophy, but did not show any beneficial influence on the number of MRI Gd enhancing lesions [134]. The Vanderblit University group is currently treating patients with MS with a combination of bacterial protein synthesis inhibitors (doxycycline and azithromycin or roxithromycin or rifampicin) and then adding metronidazole to this. These studies will need to be validated by comprehensive multicenter trials of combined antibiotic treatment aimed at all phases of the organism's life-cycle [135].

From the data presented, there is strong evidence that *C. pneumoniae* has not a causal role in MS disease. Thus, the actual involvement of *C. pneumoniae* in MS still remains a matter of debate and requires further understanding through standardized cultural, molecular and ultrastructural protocols for *C. pneumoniae* in biological samples coming from MS patients and controls. While some studies suggest a role of *C. pneumoniae* only as a CNS innocent bystander epiphenomenon due to ongoing MS inflammation which favours a selective infiltration of infected-mononuclear cells within the CNS, others indicate a role of *C. pneumoniae* as a cofactor in development and progression of the disease by enhancing a pre-existing autoimmune response in a subset of MS patients, as supported by recent immunological and molecular findings [95, 109, 136]. Recent our findings have demonstrated a possible association between *Parachlamydiae Like Organisms* and MS suggesting that these can act alone or together with *C. pneumoniae* as a cofactor in the development and progression of MS [137]. Although these data needs further assessment, their possible involvement in MS could be of great importance in public health.

Finally, we cannot exclude that other pathogens may be potentially involved in the development of MS disease. Virus have often been considered as potential candidates because they are known to cause demyelinating disease in experimental animals and man, and often cause disease with long periods of latency that presents clinically with relapsing, remitting symptoms [138]. To date, however, studies have failed to identify any single virus as playing a major role in MS. Among the virus suggested as MS cofactors, there are ubiquitous members of the family Herpesviridae, Human herpesvirus 6 (HHV-6), Epstein-Barr virus (EBV) [139–146]. As *Chlamydia*, these viruses can undergo an alternative infection cycle, entering a quiescent state (latency), with low grade viral infection that does not cause cell lysis, from which they subsequently can be reactivated. However, the cell type in which this occurs is usually not the same cell type in which the productive, cytocidal infection occurs. The human MS-associated retrovirus (MSRV) belonging to endogenous retrovirus family, has been also described as potential pathogen in MS [147].

## 4. *C. pneumoniae* and Other Neurological Complications

A number of reports have focused on the involvement of *C. pneumoniae* in other CNS disorders and in particular in encephalitis or meningoencephalitis. We searched PubMed for articles on encephalitis and *Chlamydia pneumoniae* or *Chlamydophila pneumoniae* using keywords: encephalitis, meningoencephalitis, *Chlamydia pneumoniae, Chlamydophila pneumonia,* and numerous additional keywords including "neurological complications" relevant to these topics. Due to the common usage of the previous genus name, "*Chlamydia*," it was included in the search. The reported cases (Table 3) were not so frequent [148–159]. Most patients were young patients who presented with different neurological symptoms and/or neuro-radiological changes at CT or MRI scan and in most cases, there were also well defined accompanying respiratory symptoms, although these have in some cases preceded the onset of the neurological records. Three patients had cerebellar ataxia, acute demyelinating encephalitis (ADEM), and Guillain Barrè syndrome. The detection of *Chlamydia* has been almost always done by serological methods based on detection of specific anti-*C. pneumoniae* antibodies of different classes by MIF (fourfold rise in the IgG titre) and ELISA techniques. One study detected the presence of IgA-type antibodies, suggesting a reinfection [158]. One note reported the use of PCR in a tracheal swab and increasing titres of *Chlamydia* IgM antibody [155]. These cases, and a review of the literature, suggest that *C. pneumoniae* infection in addition to

Table 3: Acute and chronic neurological complications preceding or following *C. pneumoniae* associated respiratory manifestations.

| Author, year | Patient n. | Sex/age range (yr) median | Clinical findings | *C. pneumoniae* detection | Neuroimaging | Treatment | Outcome |
|---|---|---|---|---|---|---|---|
| Fryden et al. 1989 [148] | 1 | F/16 | Encephalitis, respiratory tract infection | Serum (4-fold rise) in IgG and IgM[§] CSF negative for IgG and IgM | Brain CT scan | Clorampheni-col, Steroids | Recovery 1 year later |
| Haidl et al. 1992 [149] | 1 | M/13 | Guillan-Barrè syndrome, parestesia, hyporeflexia, cough | Serum (4-fold rise) in IgG and IgM[§] | ND | Steroids | Recovery 5 weeks later |
| Michel et al. 1992 [150] | 1 | M/9 | Meningoradicultis, cough, rhinitis, hyporeflexia, back stiffness | Serum 4-fold fall in IgM, CSF positive for total IgG | ND | ND | Recovery 6 months later |
| Sundelöf et al. 1993 [151] | 1 | M/18 | Meningoencepha-litis, headache, diplopia paresthesia, fever, cough | Serum IgG (4-fold), IgM[§] CSF negative for IgG and IgM | Brain CT scan | Erythromycin, Cefotaxime, Acyclovir | Recovery <1 week later |
| Socan et al. 1994 [152] | 1 | M/18 | Diplopia, parestesia, meningeal syndrome, cough and pneumonia | Serum IgM and IgG[§] CSF negative for IgG and IgM | Brain CT scan | Acyclovir, Cefotaxime, Erythromycin | Recovery <1 week later |
| Koskiniemi et al. 1997 [153] | 3 | F[2]/5.6 | Pareses, sensory symptoms, convulsions, depression of conscious-sness | Serum IgM and IgG[§] | Brain CT, EEG[##] | Unspecified | ND |
| Korman et al. 1997 [154] | 1 | F/69 | Cerebellar ataxia, respiratory failure | Serum (4-fold rise) in IgG, elevated IgA[§] CSF negative for IgG and IgM | Brain CT | Erythromycin, Imipenem | Recovery <1 month later |
| Heick and Skriver 2000 [155] | 1 | F/18 | Coryzal illness, headache, dizziness, left-sided hemiparesis ([†]ADEM), ataxia, fever, dry cough | CKT[#] in serum serum IgM PCR in tracheal swab | Brain CT scan, cranial MR | Penicillin, Acyclovir, Gentamycine, Cefotaxime, Doxycycline, Methylpredn-isolone | Recovery 2 months |
| Guglielminotti et al. 2000 [156] | 1 | M/95 | Meningeal syndrome, sleepiness, dry cough | Serum (4-fold rise) in IgG and IgA[§] CSF IgG | Brain CT, EEG | Acyclovir, Amoxicillin, Erythromycin, Ofloxacin | died aspiration pneumonia |
| Anton et al. 2000 [157] | 1 | F/16 | Hyporeflexia, bilateral nistagmus, sleepiness, motor weakness, rhinitis | Serum (4-fold rise) in IgG, IgM[§] CSF negative for IgG and IgM | Brain CT scan, Brain MR, Electrophy-siological studies | Ceftriaxone, Methylpredn-isolone | Recovery 2 months later |
| Airas et al. 2001 [158] | 1 | F/33 | Sleepiness, memory loss, cough, pharyngitis | Serum IgA and IgG[‡] | Brain MR | Acyclovir, Levofloxacine, Azhitromycine | Partial improvement |
| Boschin-Crinquette et al. 2005 [159] | 1 | M/21 | Aphasia, meningeal syndrome, respiratory symptoms | Serum IgM[°] CSF negative for IgM, IgG and PCR | Brain CT scan, Brain MR | Acyclovir, Ofloxacin Amoxicillin, Cefotaxime | Recovery <2 weeks later |

[§]MIF: microimmunofluorescence; [‡]EIA: enzyme immunoassay; [°]ELISA: enzyme-linked immunoadsorbent assay; [#]CTK: *Chlamydia* Complement-binding Test; [##]EEG: electroencephalogram; [†]ADEM: acute disseminated encephalomyelitis.

other *Chlamydiae*, may present with significant neurological manifestations. Of interest, most of these patients did experience a favourable outcome after administration of antibiotic therapy with or without corticosteroid treatment, suggesting a strong etiologic link between the microorganism and encephalitis. Chlamydial infections along with *Mycoplasma* and legionella infections should be included in the differential diagnosis of respiratory infections with a neurologic presentation.

## 5. *C. pneumoniae* and Neurobehavioral Disorders

Although the limited data of literature, there is evidence that *Chlamydia* may be implicated in the pathogenesis of some mental or neurobehavioral disorders including autism and schizophrenia. Autism spectrum disorders (ASDs) are a group of neurobehavioral diseases of unknown aetiology, which include autism, attention deficit disorder, Asperger's syndrome, and so forth, which causes are unknown but appear to include genetic defects, heavy metal, and chemical and biological exposures [160]. Factors, such as geography, family socioeconomic status, vaccination records, and family educational levels may be also involved. They occur primarily in the young and are probably different in each patient. Such patients do not all share the same signs and symptoms but tend to share certain social, communication, motor, and sensory problems that affect their behaviour in predictable ways. In general, the criteria for diagnosis of ASD are the presence of a triad of impairments in social interaction, communication, and imagination [160]. These signs and symptoms are thought to be due to abnormalities in brain function or structure and are thought to have a genetic basis [161, 162]. There is growing awareness that ASD can have an infectious nature that may be a cofactor for the illness or can aggravate patient morbidity [163–165]. The appearance of infections and in particular *Mycoplasma* infections in children diagnosed with ASD has been also linked to the multiple vaccines received during childhood [136, 166]. In this setting, *C. pneumoniae* [167, 168] along with a number of systemic chronic infections, such as those by *Mycoplasma* species [169–172] and HHV-6 [171–173], have been identified in Gulf War veterans and in family members including their children, using highly sensitive PCR and confirming the results by Southern-blot and dot-blot hybridization. Interestingly, a number of these symptomatic children were diagnosed with autism or attention deficit disorder that fall under ASD [174]. Based on previous observations of persisting IgA titers in some patients with mental disorders, it has been hypothesized that Chlamydiaceae are main pathogenic factors in schizophrenia. Fellerhoff, using n-PCR, found a significant prevalence of *C. psittaci*, *C. pneumonia,* and *C. trachomatis* (9/18, 50%), as compared to controls (8/115, 6.97%). Treatment with in vitro-activated immune cells together with antibiotic modalities showed sustained mental improvements in patients that did not depend on treatment with antipsychotic drugs [175].

## 6. Conclusions

*C. pneumoniae* is like a "*New Bug that's full of Surprises*" [136]. This perfectly matches to the wide range of chronic diseases which can be sustained by this pathogen. Thanks to deep knowledge of the biology of *Chlamydia* and the use of increasingly sophisticated techniques than those traditionally used, the presence of *C. pneumoniae* genomic material was demonstrated in a large number of persons suffering from different acute and chronic diseases. Over the past 10 years, a growing number of reports have found a possible link between *C. pneumoniae* infection and atherosclerosis and CNS diseases including MS, AD other than a variety of neurobehavioral disorders. The main obstacles that have so far presented to support a definitive role of *C. pneumoniae* in chronic diseases are represented by the fact that no methods exist to safely and confidently diagnose chronic infection, and because chlamydial chronic infections are characterized by the inaccessibility of the "chlamydial persistent state" to conventional antichlamydial agents. A causative role of *C. pneumoniae* infection in cardiovascular disease has not yet been firmly established. Despite the molecular and genetic efforts that have been done on the role of *C. pneumoniae* in the progression of atherosclerosis, several important questions including whether the *C. pneumoniae* is an innocent passenger or whether it is actively involved in the initiation or progression of atherosclerotic disease urgently need an answer. In particular, *C. pneumoniae* HsP60 needs to be explored further as a potential culprit and therapeutic target [11–13]. Several drugs shown to be more or less effective in atherosclerotic disease are in the recent experiments, at the same time effective against *C. pneumoniae*. Statins, aspirin, and dietary polyphenolic compounds are among them. It is possible that the truly effective treatment targeting chronic *C. pneumoniae* infection will be found. At the same time, the development of efficacious vaccine should be continued [136]. The interpretation of the fact that astrocytes, microglia, and neurons are host cells for *C. pneumoniae* in the brain of AD patients, and that infected cells can be found in close proximity to both NSP and NFT, is hampered by the fact that most studies were done with different diagnostic methods, none of which still standardized. This has led a wide variation of interlaboratory test performance, even when the same test and the same criteria have been used. Thus, the actual involvement of *C. pneumoniae* in AD still remains a matter of debate and requires further understanding through standardized cultural, molecular protocols for *C. pneumoniae* in autoptic samples coming from AD patients and controls [51–53, 70]. The recent molecular, ultrastructural, and cultural advances that have provided evidence that *C. pneumoniae* is viable and metabolically active in different biological compartments such as CSF and PBMC from MS patients compared to controls, suggests an association between this pathogen and the disease, particularly in a subgroup of RR MS patients with clinical and MRI disease activity who experience the early inflammatory phase representing the development of the disease [89, 95, 108, 109]. However, the growing body of evidence suggests a role of *C. pneumoniae* only as a CNS innocent bystander

epiphenomenon due to ongoing MS inflammation or a cofactor in development and progression of the disease by enhancing a pre-existing autoimmune response in a subset of MS patients, as supported by the recent immunological and molecular findings [95, 96, 109]. Either for AD or MS there is urgency for further well-designed studies to determine both the importance of *C. pneumoniae* involvement in human diseases and the usefulness of antibiotic treatment. The role of *Chlamydia* in the pathogenesis of mental or neurobehavioral disorders including schizophrenia and autism is uncertain and fragmentary. However, the few existing reports suggest a potential involvement which will require further confirmation.

## Acknowledgment

This work was in part supported by the Research Program Regione Emilia Romagna—University 2007–2009 (Innovative Research), entitled: "Regional Network for Implementing a Bank to Identify Biological Markers of Activity Disease to Clinical Variables in Multiple Sclerosis."

## References

[1] K. D. E. Everett, R. M. Bush, and A. A. Andersen, "Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms," *International Journal of Systematic Bacteriology*, vol. 49, no. 2, pp. 415–440, 1999.

[2] P. Saikku, M. Leinonen, K. Mattila, et al., "Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction," *The Lancet*, vol. 2, no. 8618, pp. 983–986, 1988.

[3] M. Kleemola, P. Saikku, R. Visakorpi, S. P. Wang, and J. T. Grayston, "Epidemics of pneumonia caused by TWAR, a new *Chlamydia* organism, in military trainees in Finland," *Journal of Infectious Diseases*, vol. 157, no. 2, pp. 230–236, 1988.

[4] C.-C. Kuo, L. A. Jackson, L. A. Campbell, and J. T. Grayston, "*Chlamydia pneumoniae* (TWAR)," *Clinical Microbiology Reviews*, vol. 8, no. 4, pp. 451–461, 1995.

[5] C. H. Mordhorst, S. P. Wang, and J. T. Grayston, "Outbreak of *Chlamydia pneumoniae* infection in four farm families," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 11, no. 7, pp. 617–620, 1992.

[6] C. Yucesan and S. Sriram, "*Chlamydia pneumoniae* infection of the central nervous system," *Current Opinion in Neurology*, vol. 14, no. 3, pp. 355–359, 2001.

[7] C. W. Stratton and S. Sriram, "Association of *Chlamydia pneumoniae* with central nervous system disease," *Microbes and Infection*, vol. 5, no. 13, pp. 1249–1253, 2003.

[8] C. W. Stratton and W. M. Mitchell, "The immunopathology of chlamydial infections," *Antimicrobics and Infectious Diseases Newsletter*, vol. 16, no. 12, pp. 89–94, 1997.

[9] A. Ciervo, P. Visca, A. Petrucca, L. M. Biasucci, A. Maseri, and A. Cassone, "Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of *Chlamydia pneumoniae* in patients with coronary heart disease," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 1, pp. 66–74, 2002.

[10] O. S. Mahdi, B. D. Horne, K. Mullen, J. B. Muhlestein, and G. I. Byrne, "Serum immunoglobulin G antibodies to chlamydial heat shock protein 60 but not to human and bacterial homologs are associated with coronary artery disease," *Circulation*, vol. 106, no. 13, pp. 1659–1663, 2002.

[11] M. Leinonen and P. Saikku, "Evidence for infectious agents in cardiovascular disease and atherosclerosis," *Lancet Infectious Diseases*, vol. 2, no. 1, pp. 11–17, 2002.

[12] L. A. Campbell and C.-C. Kuo, "*Chlamydia pneumoniae*: an infectious risk factor for atherosclerosis?" *Nature Reviews Microbiology*, vol. 2, no. 1, pp. 23–32, 2004.

[13] F. F. Mussa, H. Chai, X. Wang, Q. Yao, A. B. Lumsden, and C. Chen, "*Chlamydia pneumoniae* and vascular disease: an update," *Journal of Vascular Surgery*, vol. 43, no. 6, pp. 1301–1307, 2006.

[14] A. Palikhe, M.-L. Lokki, P. Saikku, et al., "Association of *Chlamydia pneumoniae* infection with HLA-B*35 in patients with coronary artery disease," *Clinical and Vaccine Immunology*, vol. 15, no. 1, pp. 55–59, 2008.

[15] G. I. Byrne, "*Chlamydia* uncloaked," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8040–8042, 2003.

[16] G. I. Byrne and D. M. Ojcius, "*Chlamydia* and apoptosis: life and death decisions of an intracellular pathogen," *Nature Reviews Microbiology*, vol. 2, no. 10, pp. 802–808, 2004.

[17] J. Boman, S. Söderberg, J. Forsberg, et al., "High prevalence of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors," *Journal of Infectious Diseases*, vol. 178, no. 1, pp. 274–277, 1998.

[18] A. MacIntyre, R. Abramov, C. J. Hammond, et al., "*Chlamydia pneumoniae* infection promotes the transmigration of monocytes through human brain endothelial cells," *Journal of Neuroscience Research*, vol. 71, no. 5, pp. 740–750, 2003.

[19] C. C. Kuo and L. A. Campbell, "Chlamydial infections of the cardiovascular system," *Frontiers in Bioscience*, vol. 8, pp. e36–e43, 2003.

[20] R. W. Peeling, S.-P. Wang, J. T. Grayston, et al., "*Chlamydia pneumoniae* serology: interlaboratory variation in microimmunofluorescence assay results," *Journal of Infectious Diseases*, vol. 181, no. 6, supplement 3, pp. S426–S429, 2000.

[21] P. Apfalter, "*Chlamydia pneumoniae*, stroke, and serological associations: anything learned from the atherosclerosis-cardiovascular literature or do we have to start over again?" *Stroke*, vol. 37, no. 3, pp. 756–758, 2006.

[22] N. Mackman, "Lipopolysaccharide induction of gene expression in human monocytic cells," *Immunologic Research*, vol. 21, no. 2-3, pp. 247–251, 2000.

[23] M. V. Kalayoglu, L. Indrawati, R. P. Morrison, S. G. Morrison, Y. Yuan, and G. I. Byrne, "Chlamydial virulence determinants in atherogenesis: the role of chlamydial lipopolysaccharide and heat shock protein 60 in macrophage-lipoprotein interactions," *Journal of Infectious Diseases*, vol. 181, no. 6, supplement 3, pp. S483–S489, 2000.

[24] I. W. Fong, B. Chiu, E. Viira, W. Tucker, H. Wood, and R. W. Peeling, "Chlamydial heat-shock protein-60 antibody and correlation with *Chlamydia pneumoniae* in atherosclerotic plaques," *Journal of Infectious Diseases*, vol. 186, no. 10, pp. 1469–1473, 2002.

[25] T. Huittinen, M. Leinonen, L. Tenkanen, et al., "Autoimmunity to human heat shock protein 60, *Chlamydia pneumoniae* infection, and inflammation in predicting coronary risk," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 3, pp. 431–437, 2002.

[26] M. L. J. Wimmer, R. Sandmann-Strupp, P. Saikku, and R. L. Haberl, "Association of chlamydial infection with cerebrovascular disease," *Stroke*, vol. 27, no. 12, pp. 2207–2210, 1996.

[27] M. S. V. Elkind, I.-F. Lin, J. T. Grayston, and R. L. Sacco, "*Chlamydia pneumoniae* and the risk of first ischemic stroke: the Northern Manhattan Stroke Study," *Stroke*, vol. 31, no. 7, pp. 1521–1525, 2000.

[28] B. Piechowski-Jóźwiak, A. Mickielewicz, Z. Gaciong, H. Berent, and H. Kwieciński, "Elevated levels of anti-*Chlamydia pneumoniae* IgA and IgG antibodies in young adults with ischemic stroke," *Acta Neurologica Scandinavica*, vol. 116, no. 3, pp. 144–149, 2007.

[29] J. G. Madre, J. L. R. Garcia, R. C. Gonzalez, et al., "Association between seropositivity to *Chlamydia pneumoniae* and acute ischaemic stroke," *European Journal of Neurology*, vol. 9, no. 3, pp. 303–306, 2002.

[30] M. S. V. Elkind, M. L. C. Tondella, D. R. Feikin, B. S. Fields, S. Homma, and M. R. Di Tullio, "Seropositivity to *Chlamydia pneumoniae* is associated with risk of first ischemic stroke," *Stroke*, vol. 37, no. 3, pp. 790–795, 2006.

[31] P. U. Heuschmann, D. Neureiter, M. Gesslein, et al., "Association between infection with *Helicobacter pylori* and *Chlamydia pneumoniae* and risk of ischemic stroke subtypes: results from a population-based case-control study," *Stroke*, vol. 32, no. 10, pp. 2253–2258, 2001.

[32] A. Anzini, A. Cassone, M. Rasura, et al., "*Chlamydia pneumoniae* infection in young stroke patients: a case-control study," *European Journal of Neurology*, vol. 11, no. 5, pp. 321–327, 2004.

[33] A. Vink, M. Poppen, A. H. Schoneveld, et al., "Distribution of *Chlamydia pneumoniae* in the human arterial system and its relation to the local amount of atherosclerosis within the individual," *Circulation*, vol. 103, no. 12, pp. 1613–1617, 2001.

[34] K. Numazaki and S. Chibar, "Failure to detect *Chlamydia pneumoniae* in the central nervous system of patients with MS," *Neurology*, vol. 57, no. 4, p. 746, 2001.

[35] S. F. Dowell, R. W. Peeling, J. Boman, et al., "Standardizing *Chlamydia pneumoniae* assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada)," *Clinical Infectious Diseases*, vol. 33, no. 4, pp. 492–502, 2001.

[36] J. Boman and M. R. Hammerschlag, "*Chlamydia pneumoniae* and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies," *Clinical Microbiology Reviews*, vol. 15, no. 1, pp. 1–20, 2002.

[37] M. M. Ieven and V. Y. Hoymans, "Involvement of *Chlamydia pneumoniae* in atherosclerosis: more evidence for lack of evidence," *Journal of Clinical Microbiology*, vol. 43, no. 1, pp. 19–24, 2005.

[38] A. J. Littman, L. A. Jackson, E. White, M. D. Thornquist, C. A. Gaydos, and T. L. Vaughan, "Interlaboratory reliability of microimmunofluorescence test for measurement of *Chlamydia pneumoniae*-specific immunoglobulin A and G antibody titers," *Clinical and Diagnostic Laboratory Immunology*, vol. 11, no. 3, pp. 615–617, 2004.

[39] P. Maggi, R. Monno, A. Chirianni, et al., "Role of *Chlamydia* infection in the pathogenesis of atherosclerotic plaques in HIV-1-positive patients," *In Vivo*, vol. 20, no. 3, pp. 409–414, 2006.

[40] G. Tositti, M. Rassu, P. Fabris, et al., "*Chlamydia pneumoniae* infection in HIV-positive patients: prevalence and relationship with lipid profile," *HIV Medicine*, vol. 6, no. 1, pp. 27–32, 2005.

[41] V. Gaona-Flores, G. García-Elorriaga, M. Valerio-Minero, et al., "Anti-Chlamydophila pneumoniae antibodies as associated factor for carotoid atherosclerosis in patients wit AIDS," *Current HIV Research*, vol. 6, no. 3, pp. 267–271, 2008.

[42] P. Apfalter, O. Assadian, F. Blasi, et al., "Reliability of nested PCR for detection of *Chlamydia pneumoniae* DNA in atheromas: results from a multicenter study applying standardized protocols," *Journal of Clinical Microbiology*, vol. 40, no. 12, pp. 4428–4434, 2002.

[43] R. Sessa, M. Di Pietro, G. Schiavoni, et al., "*Chlamydia pneumoniae* DNA in patients with symptomatic carotid atherosclerotic disease," *Journal of Vascular Surgery*, vol. 37, no. 5, pp. 1027–1031, 2003.

[44] A. Palikhe, T. Tiirola, M. Puolakkainen, et al., "*Chlamydia pneumoniae* DNA is present in peripheral blood mononuclear cells during acute coronary syndrome and correlates with chlamydial lipopolysaccharide levels in serum," *Scandinavian Journal of Infectious Diseases*, vol. 41, no. 3, pp. 201–205, 2009.

[45] J. Gieffers, H. Füllgraf, J. Jahn, et al., "*Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment," *Circulation*, vol. 103, no. 3, pp. 351–356, 2001.

[46] J. Gieffers, J. Rupp, A. Gebert, W. Solbach, and M. Klinger, "First-choice antibiotics at subinhibitory concentrations induce persistence of *Chlamydia pneumoniae*," *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 4, pp. 1402–1405, 2004.

[47] J. T. Grayston, R. A. Kronmal, L. A. Jackson, et al., "Azithromycin for the secondary prevention of coronary events," *The New England Journal of Medicine*, vol. 352, no. 16, pp. 1637–1645, 2005.

[48] B. J. Balin, H. C. Gérard, E. J. Arking, et al., "Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain," *Medical Microbiology and Immunology*, vol. 187, no. 1, pp. 23–42, 1998.

[49] D. Nochlin, C. M. Shaw, L. A. Campbell, and C.-C. Kuo, "Failure to detect *Chlamydia pneumoniae* in brain tissues of Alzheimer's disease," *Neurology*, vol. 53, no. 8, p. 1888, 1999.

[50] J. Gieffers, E. Reusche, W. Solbach, and M. Maass, "Failure to detect *Chlamydia pneumoniae* in brain sections of Alzheimer's disease patients," *Journal of Clinical Microbiology*, vol. 38, no. 2, pp. 881–882, 2000.

[51] R. H. Ring and J. M. Lyons, "Failure to detect *Chlamydia pneumoniae* in the late-onset Alzheimer's brain," *Journal of Clinical Microbiology*, vol. 38, no. 7, pp. 2591–2594, 2000.

[52] G. S. Taylor, I. B. Vipond, I. D. Paul, S. Matthews, G. K. Wilcock, and E. O. Caul, "Failure to correlate *C. pneumoniae* with late onset Alzheimer's disease," *Neurology*, vol. 59, no. 1, pp. 142–143, 2002.

[53] M. A. Wozniak, A. Cookson, G. K. Wilcock, and R. F. Itzhaki, "Absence of *Chlamydia pneumoniae* in brain of vascular dementia patients," *Neurobiology of Aging*, vol. 24, no. 6, pp. 761–765, 2003.

[54] H. C. Gérard, U. Dreses-Werringloer, K. S. Wildt, et al., "Chlamydophila (*Chlamydia*) *pneumoniae* in the Alzheimer's brain," *FEMS Immunology and Medical Microbiology*, vol. 48, no. 3, pp. 355–366, 2006.

[55] B. J. Balin and D. M. Appelt, "Role of infection in Alzheimer's disease," *Journal of the American Osteopathic Association*, vol. 101, no. 12, supplement 1, pp. S1–S6, 2001.

[56] R. W. Keefover, "The clinical epidemiology of Alzheimer's disease," *Neurologic Clinics*, vol. 14, no. 2, pp. 337–351, 1996.

[57] C. Kawas, S. Gray, R. Brookmeyer, J. Fozard, and A. Zonderman, "Age-specific incidence rates of Alzheimer's disease: the Baltimore Longitudinal Study of Aging," *Neurology*, vol. 54, no. 11, pp. 2072–2077, 2000.

[58] A. Di Carlo, M. Baldereschi, L. Amaducci, et al., "Incidence of dementia, Alzheimer's disease, and vascular dementia in Italy. The ILSA study," *Journal of the American Geriatrics Society*, vol. 50, no. 1, pp. 41–48, 2002.

[59] G. Ravaglia, P. Forti, F. Maioli, et al., "Incidence and etiology of dementia in a large elderly Italian population," *Neurology*, vol. 64, no. 9, pp. 1525–1530, 2005.

[60] D. A. Evans, D. A. Bennett, R. S. Wilson, et al., "Incidence of Alzheimer disease in a biracial urban community: relation to apolipoprotein E allele status," *Archives of Neurology*, vol. 60, no. 2, pp. 185–189, 2003.

[61] R. Brookmeyer, S. Gray, and C. Kawas, "Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset," *American Journal of Public Health*, vol. 88, no. 9, pp. 1337–1342, 1998.

[62] L.-F. Lue, L. Brachova, W. H. Civin, and J. Rogers, "Inflammation, A$\beta$ deposition, and neurofibrillary tangle formation as correlates of Alzheimer's disease neurodegeneration," *Journal of Neuropathology and Experimental Neurology*, vol. 55, no. 10, pp. 1083–1088, 1996.

[63] R. F. Itzhaki, W.-R. Lin, D. Shang, G. K. Wilcock, B. Faragher, and G. A. Jamieson, "Herpes simplex virus type 1 in brain and risk of Alzheimer's disease," *The Lancet*, vol. 349, no. 9047, pp. 241–244, 1997.

[64] R. F. Itzhaki, C. B. Dobson, W.-R. Lin, and M. A. Wozniak, "Association of HSV1 and apolipoprotein E-$\varepsilon$4 in Alzheimer's disease," *Journal of NeuroVirology*, vol. 7, no. 6, pp. 570–571, 2001.

[65] B. G. T. Pogo, J. Casals, and T. S. Elizan, "A study of viral genomes and antigens in brains of patients with Alzheimer's disease," *Brain*, vol. 110, no. 4, pp. 907–915, 1987.

[66] R. P. Friedland, C. May, and J. Dahlberg, "The viral hypothesis of Alzheimer's disease: absence of antibodies to lentiviruses," *Archives of Neurology*, vol. 47, no. 2, pp. 177–178, 1990.

[67] E. B. Renvoize, I. O. Awad, and M. H. Hambling, "A sero-epidemiological study of conventional infectious agents in Alzheimer's disease," *Age and Ageing*, vol. 16, no. 5, pp. 311–314, 1987.

[68] J. Miklossy, "Alzheimer's disease—a spirochetosis?" *NeuroReport*, vol. 4, no. 7, pp. 841–848, 1993.

[69] W. B. Matthews, "Unconventional virus infection and neurological disease," *Neuropathology and Applied Neurobiology*, vol. 12, no. 2, pp. 111–116, 1986.

[70] S. R. Robinson, C. Dobson, and J. Lyons, "Challenges and directions for the pathogen hypothesis of Alzheimer's disease," *Neurobiology of Aging*, vol. 25, no. 5, pp. 629–637, 2004.

[71] U. Dreses-Werringloer, M. Bhuiyan, Y. Zhao, H. C. Gérard, J. A. Whittum-Hudson, and A. P. Hudson, "Initial characterization of *Chlamydophila* (*Chlamydia*) *pneumoniae* cultured from the late-onset Alzheimer brain," *International Journal of Medical Microbiology*, vol. 299, no. 3, pp. 187–201, 2009.

[72] H. C. Gérard, G. F. Wang, B. J. Balin, H. R. Schumacher, and A. P. Hudson, "Frequency of apolipoprotein E (APOE) allele types in patients with *Chlamydia*-associated arthritis and other arthritides," *Microbial Pathogenesis*, vol. 26, no. 1, pp. 35–43, 1999.

[73] H. C. Gérard, K. L. Wildt, J. A. Whittum-Hudson, Z. Lai, J. Ager, and A. P. Hudson, "The load of *Chlamydia pneumoniae* in the Alzheimer's brain varies with APOE genotype," *Microbial Pathogenesis*, vol. 39, no. 1-2, pp. 19–26, 2005.

[74] A. MacIntyre, C. J. Hammond, C. S. Little, D. M. Appelt, and B. J. Balin, "*Chlamydia pneumoniae* infection alters the junctional complex proteins of human brain microvascular endothelial cells," *FEMS Microbiology Letters*, vol. 217, no. 2, pp. 167–172, 2002.

[75] C. S. Little, C. J. Hammond, A. MacIntyre, B. J. Balin, and D. M. Appelt, "*Chlamydia pneumoniae* induces Alzheimer-like amyloid plaques in brains of BALB/c mice," *Neurobiology of Aging*, vol. 25, no. 4, pp. 419–429, 2004.

[76] M. B. Loeb, D. W. Molloy, M. Smieja, et al., "A randomized, controlled trial of doxycycline and rifampin for patients with Alzheimer's disease," *Journal of the American Geriatrics Society*, vol. 52, no. 3, pp. 381–387, 2004.

[77] A. E. Finefrock, A. I. Bush, and P. M. Doraiswamy, "Current status of metals as therapeutic targets in Alzheimer's disease," *Journal of the American Geriatrics Society*, vol. 51, no. 8, pp. 1143–1148, 2003.

[78] L. Cahoon, "The curious case of clioquinol," *Nature Medicine*, vol. 15, no. 4, pp. 356–359, 2009.

[79] C. Contini, E. Fainardi, S. Seraceni, E. Granieri, M. Castellazzi, and R. Cultrera, "Molecular identification and antibody testing of *Chlamydophila pneumoniae* in a subgroup of patients with HIV-associated dementia complex. Preliminary results," *Journal of Neuroimmunology*, vol. 136, no. 1-2, pp. 172–177, 2003.

[80] S. Gartner, "HIV infection and dementia," *Science*, vol. 287, no. 5453, pp. 602–604, 2000.

[81] G. Layh-Schmitt, C. Bendl, U. Hildt, et al., "Evidence for infection with *Chlamydia pneumoniae* in a subgroup of patients with multiple sclerosis," *Annals of Neurology*, vol. 47, no. 5, pp. 652–655, 2000.

[82] W. P. Li, X. Ming, S. Cook, B. Blumberg, and P. Dowling, "*Chlamydia pneumoniae* sequence frequently present in both MS and control spinal fluid," *Neurology*, vol. 54, supplement 3, p. A165, 2000.

[83] J. Treib, A. Haaß, W. Stille, et al., "Multiple sclerosis and *Chlamydia pneumoniae*," *Annals of Neurology*, vol. 47, no. 3, p. 408, 2000.

[84] J. Gieffers, D. Pohl, J. Treib, et al., "Presence of *Chlamydia pneumoniae* DNA in the cerebral spinal fluid is a common phenomenon in a variety of neurological diseases and not restricted to multiple sclerosis," *Annals of Neurology*, vol. 49, no. 5, pp. 585–589, 2001.

[85] S. Sotgiu, A. Piana, M. Pugliatti, et al., "*Chlamydia pneumoniae* in the cerebrospinal fluid of patients with multiple sclerosis and neurological controls," *Multiple Sclerosis*, vol. 7, no. 6, pp. 371–374, 2001.

[86] H. Ikejima, S. Haranaga, H. Takemura, et al., "PCR-based method for isolation and detection of *Chlamydia pneumoniae* DNA in cerebrospinal fluids," *Clinical and Diagnostic Laboratory Immunology*, vol. 8, no. 3, pp. 499–502, 2001.

[87] Q. Hao, N. Miyashita, M. Matsui, H.-Y. Wang, T. Matsushima, and T. Saida, "*Chlamydia pneumoniae* infection associated with enhanced MRI spinal lesions in multiple sclerosis," *Multiple Sclerosis*, vol. 8, no. 5, pp. 436–440, 2002.

[88] S. Chatzipanagiotou, C. Tsakanikas, M. Anagnostouli, M. Rentzos, A. Ioannidis, and C. Nicolaou, "Detection of *Chlamydia pneumoniae* in the cerebrospinal fluid of patients

with multiple sclerosis by combination of cell culture and PCR. No evidence for possible association," *Molecular Diagnosis*, vol. 7, no. 1, pp. 41–43, 2003.

[89] L. M. E. Grimaldi, A. Pincherle, F. Martinelli-Boneschi, et al., "An MRI study of *Chlamydia pneumoniae* infection in Italian multiple sclerosis patients," *Multiple Sclerosis*, vol. 9, no. 5, pp. 467–471, 2003.

[90] K. Rostasy, H. Reiber, D. Pohl, et al., "*Chlamydia pneumoniae* in children with MS: frequency and quantity of intrathecal antibodies," *Neurology*, vol. 61, no. 1, pp. 125–128, 2003.

[91] T. Dong-Si, J. Weber, Y. B. Liu, et al., "Increased prevalence of and gene transcription by *Chlamydia pneumoniae* in cerebrospinal fluid of patients with relapsing-remitting multiple sclerosis," *Journal of Neurology*, vol. 251, no. 5, pp. 542–547, 2004.

[92] C. Contini, R. Cultrera, S. Seraceni, M. Castellazzi, E. Granieri, and E. Fainardi, "Cerebrospinal fluid molecular demonstration of *Chlamydia pneumoniae* DNA is associated to clinical and brain magnetic resonance imaging activity in a subset of patients with relapsing-remitting multiple sclerosis," *Multiple Sclerosis*, vol. 10, no. 4, pp. 360–369, 2004.

[93] S. Sriram, A. Ljunggren-Rose, S.-Y. Yao, and W. O. Whetsell Jr., "Detection of chlamydial bodies and antigens in the central nervous system of patients with multiple sclerosis," *Journal of Infectious Diseases*, vol. 192, no. 7, pp. 1219–1228, 2005.

[94] R. Sessa, G. Schiavoni, G. Borriello, et al., "Real time PCR for detection of *Chlamydophila pneumoniae* in peripheral blood mononuclear cells of patients with multiple sclerosis," *Journal of Neurology*, vol. 254, no. 9, pp. 1293–1295, 2007.

[95] C. Contini, S. Seraceni, M. Castellazzi, E. Granieri, and E. Fainardi, "*Chlamydophila pneumoniae* DNA and mRNA transcript levels in peripheral blood mononuclear cells and cerebrospinal fluid of patients with multiple sclerosis," *Neuroscience Research*, vol. 62, no. 1, pp. 58–61, 2008.

[96] Y.-W. Tang, S. Sriram, H. Li, et al., "Qualitative and quantitative detection of *Chlamydophila pneumoniae* DNA in cerebrospinal fluid from multiple sclerosis patients and controls," *PLoS ONE*, vol. 4, no. 4, article e5200, 2009.

[97] D. W. Anderson, J. H. Ellenberg, C. M. Leventhal, S. C. Reingold, M. Rodriguez, and D. H. Silberberg, "Revised estimate of the prevalence of multiple sclerosis in the United States," *Annals of Neurology*, vol. 31, no. 3, pp. 333–336, 1992.

[98] M. H. Barnett and I. Sutton, "The pathology of multiple sclerosis: a paradigm shift," *Current Opinion in Neurology*, vol. 19, no. 3, pp. 242–247, 2006.

[99] J. W. Princeas, E. E. Kwon, E.-S. Cho, and L. R. Sharer, "Continual breakdown and regeneration of myelin in progressive multiple sclerosis plaques," *Annals of the New York Academy of Sciences*, vol. 436, pp. 11–32, 1984.

[100] M. Bsibsi, R. Ravid, D. Gveric, and J. M. van Noort, "Broad expression of Toll-like receptors in the human central nervous system," *Journal of Neuropathology and Experimental Neurology*, vol. 61, no. 11, pp. 1013–1021, 2002.

[101] A. G. Joyee and X. Yang, "Role of toll-like receptors in immune responses to chlamydial infections," *Current Pharmaceutical Design*, vol. 14, no. 6, pp. 593–600, 2008.

[102] S. Seraceni, L. Badia, A. Grilli, E. Fainardi, and C. Contini, "Role of toll like receptors in patients with multiple sclerosis and *Chlamydophila pneumoniae* infection," in *Proceedings of the European Congress of Clinical Microbiology and Infectious Diseases*, Helsinki, Finland, May 2009, abstract no. R2174.

[103] D. H. Gilden, "Infectious causes of multiple sclerosis," *Lancet Neurology*, vol. 4, no. 3, pp. 195–202, 2005.

[104] I. Steiner, P. Nisipianu, and I. Wirguin, "Infection and the etiology and pathogenesis of multiple sclerosis," *Current Neurology and Neuroscience Reports*, vol. 1, no. 3, pp. 271–276, 2001.

[105] D. H. Gilden, "*Chlamydia*: a role for multiple sclerosis or more confusion," *Annals of Neurology*, vol. 46, no. 1, pp. 4–5, 1999.

[106] S. Sriram, W. Mitchell, and C. Stratton, "Multiple sclerosis associated with *Chlamydia pneumoniae* infection of the CNS," *Neurology*, vol. 50, no. 2, pp. 571–572, 1998.

[107] S. Sriram, C. W. Stratton, S.-Y. Yao, et al., "*Chlamydia pneumoniae* infection of the central nervous system in multiple sclerosis," *Annals of Neurology*, vol. 46, no. 1, pp. 6–14, 1999.

[108] C. W. Stratton and D. B. Wheldon, "Multiple sclerosis: an infectious syndrome involving *Chlamydophila pneumoniae*," *Trends in Microbiology*, vol. 14, no. 11, pp. 474–479, 2006.

[109] E. Fainardi, M. Castellazzi, S. Seraceni, E. Granieri, and C. Contini, "Under the microscope: focus on *Chlamydia pneumoniae* infection and multiple sclerosis," *Current Neurovascular Research*, vol. 5, no. 1, pp. 60–70, 2008.

[110] K. L. Munger, R. W. Peeling, M. A. Hernán, et al., "Infection with *Chlamydia pneumoniae* and risk of multiple sclerosis," *Epidemiology*, vol. 14, no. 2, pp. 141–147, 2003.

[111] K. L. Munger, G. N. DeLorenze, L. I. Levin, et al., "A prospective study of *Chlamydia pneumoniae* infection and risk of MS in two US cohorts," *Neurology*, vol. 62, no. 10, pp. 1799–1803, 2004.

[112] D. Buljevac, R. P. Verkooyen, B. C. Jacobs, et al., "*Chlamydia pneumoniae* and the risk for exacerbation in multiple sclerosis patients," *Annals of Neurology*, vol. 54, no. 6, pp. 828–831, 2003.

[113] M. R. Hammerschlag, Z. Ke, F. Lu, P. Roblin, J. Boman, and B. Kalman, "Is *Chlamydia pneumoniae* present in brain lesions of patients with multiple sclerosis?" *Journal of Clinical Microbiology*, vol. 38, no. 11, pp. 4274–4276, 2000.

[114] J. Boman, P. M. Roblin, P. Sundström, M. Sandström, and M. R. Hammerschlag, "Failure to detect *Chlamydia pneumoniae* in the central nervous system of patients with MS," *Neurology*, vol. 54, no. 1, p. 265, 2000.

[115] S. D. Poland and G. P. A. Rice, "*Chlamydia pneumoniae* and multiple sclerosis," *Neurology*, vol. 54, supplement 3, p. A165, 2000.

[116] S. J. Furrows, J. C. Hartley, J. Bell, et al., "*Chlamydophila pneumoniae* infection of the central nervous system in patients with multiple sclerosis," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 75, no. 1, pp. 152–154, 2004.

[117] F. Cirino, W. C. Webley, C. West, N. L. Croteau, C. Andrezejewski Jr., and E. S. Stuart, "Detection of *Chlamydia* in the peripheral blood cells of normal donors using in vitro culture, immunofluorescence microscopy and flow cytometry techniques," *BMC Infectious Diseases*, vol. 6, article 23, 2006.

[118] Y. Yamamoto, "PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 3, pp. 508–514, 2002.

[119] J. B. Mahony, S. Chong, B. K. Coombes, M. Smieja, and A. Petrich, "Analytical sensitivity, reproducibility of results, and clinical performance of five PCR assays for detecting *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells," *Journal of Clinical Microbiology*, vol. 38, no. 7, pp. 2622–2627, 2000.

[120] M. Kaufman, C. A. Gaydos, S. Sriram, J. Boman, M. L. Tondella, and H. J. Norton, "Is *Chlamydia pneumoniae*

found in spinal fluid samples from multiple sclerosis patients? Conflicting results," *Multiple Sclerosis*, vol. 8, no. 4, pp. 289–294, 2002.

[121] S. Sriram, S.-Y. Yao, C. Stratton, et al., "Comparative study of the presence of *Chlamydia pneumoniae* in cerebrospinal fluid of patients with clinically definite and monosymptomatic multiple sclerosis," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 6, pp. 1332–1337, 2002.

[122] A. Saiz, M. A. Marcos, F. Graus, J. Vidal, and M. T. Jimenez de Anta, "No evidence of CNS infection with *Chlamydia pneumoniae* in patients with multiple sclerosis," *Journal of Neurology*, vol. 248, no. 7, pp. 617–618, 2001.

[123] T. Derfuss, R. Gürkov, F. T. Bergh, et al., "Intrathecal antibody production against *Chlamydia pneumoniae* in multiple sclerosis is part of a polyspecific immune response," *Brain*, vol. 124, no. 7, pp. 1325–1335, 2001.

[124] F. Budak, S. Keçeli, H. Efendi, F. Budak, and H. Vahaboğlu, "The investigation of *Chlamydophila pneumoniae* in patients with multiple sclerosis," *International Journal of Neuroscience*, vol. 117, no. 3, pp. 409–415, 2007.

[125] S. A. Morré, C. J. A. de Groot, J. Killestein, et al., "Is *Chlamydia pneumoniae* present in the central nervous system of multiple sclerosis patients?" *Annals of Neurology*, vol. 48, no. 3, p. 399, 2000.

[126] E. Pucci, C. Taus, E. Cartechini, et al., "Lack of *Chlamydia* infection of the central nervous system in multiple sclerosis," *Annals of Neurology*, vol. 48, no. 3, pp. 399–400, 2000.

[127] E. J. Thompson and M. S. Freedman, "Cerebrospinal fluid analysis in the diagnosis of multiple sclerosis," *Advances in Neurology*, vol. 98, pp. 147–160, 2006.

[128] T. Smith-Jensen, M. P. Burgoon, J. Anthony, H. Kraus, D. H. Gilden, and G. P. Owens, "Comparison of immunoglobulin G heavy-chain sequences in MS and SSPE brains reveals an antigen-driven response," *Neurology*, vol. 54, no. 6, pp. 1227–1232, 2000.

[129] D. Krametter, G. Niederwieser, A. Berghold, et al., "*Chlamydia pneumoniae* in multiple sclerosis: humoral immune responses in serum and cerebrospinal fluid and correlation with disease activity marker," *Multiple Sclerosis*, vol. 7, no. 1, pp. 13–18, 2001.

[130] D. Franciotta, E. Zardini, R. Bergamaschi, L. M. Grimaldi, L. Andreoni, and V. Cosi, "Analysis of *Chlamydia pneumoniae*-specific oligoclonal bands in multiple sclerosis and other neurologic diseases," *Acta Neurologica Scandinavica*, vol. 112, no. 4, pp. 238–241, 2005.

[131] E. Fainardi, M. Castellazzi, I. Casetta, et al., "Intrathecal production of *Chlamydia pneumoniae*-specific high-affinity antibodies is significantly associated to a subset of multiple sclerosis patients with progressive forms," *Journal of the Neurological Sciences*, vol. 217, no. 2, pp. 181–188, 2004.

[132] P. G. Bagos, G. Nikolopoulos, and A. Ioannidis, "*Chlamydia pneumoniae* infection and the risk of multiple sclerosis: a meta-analysis," *Multiple Sclerosis*, vol. 12, no. 4, pp. 397–411, 2006.

[133] L. M. Metz, Y. Zhang, M. Yeung, et al., "Minocycline reduces gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis," *Annals of Neurology*, vol. 55, no. 5, p. 756, 2004.

[134] S. Sriram, S. Y. Yao, C. Stratton, H. Moses, P. A. Narayana, and J. S. Wolinsky, "Pilot study to examine the effect of antibiotic therapy on MRI outcomes in RRMS," *Journal of the Neurological Sciences*, vol. 234, no. 1-2, pp. 87–91, 2005.

[135] C. W. Stratton and D. B. Wheldon, "Antimicrobial treatment of multiple sclerosis," *Infection*, vol. 35, no. 5, pp. 383–385, 2007.

[136] C. Contini, "International conference on chlamydial and *Mycoplasma* human infections," *Future Microbiology*, vol. 2, no. 4, pp. 373–376, 2007.

[137] C. Contini, S. Seraceni, R. Cultrera, M. Castellazzi, E. Granieri, and E. Fainardi, "Molecular detection of *Parachlamydia*-like organisms in cerebrospinal fluid of patients with multiple sclerosis," *Multiple Sclerosis*, vol. 14, no. 4, pp. 564–566, 2008.

[138] D. H. Gilden, M. E. Devlin, M. P. Burgoon, and G. P. Owens, "The search for virus in multiple sclerosis brain," *Multiple Sclerosis*, vol. 2, no. 4, pp. 179–183, 1996.

[139] R. Álvarez-Lafuente, V. De las Heras, M. Bartolomé, J. J. Picazo, and R. Arroyo, "Relapsing-remitting multiple sclerosis and human herpesvirus 6 active infection," *Archives of Neurology*, vol. 61, no. 10, pp. 1523–1527, 2004.

[140] F. G. A. Moore and C. Wolfson, "Human herpes virus 6 and multiple sclerosis," *Acta Neurologica Scandinavica*, vol. 106, no. 2, pp. 63–83, 2002.

[141] T. Christensen, "The role of EBV in MS pathogenesis," *International MS Journal*, vol. 13, no. 2, pp. 52–57, 2006.

[142] D. Clark, "Human herpesvirus type 6 and multiple sclerosis," *Herpes*, vol. 11, supplement 2, pp. 112A–119A, 2004.

[143] G. N. DeLorenze, K. L. Munger, E. T. Lennette, N. Orentreich, J. H. Vogelman, and A. Ascherio, "Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up," *Archives of Neurology*, vol. 63, no. 6, pp. 839–844, 2006.

[144] P. Höllsberg, M. Kusk, E. Bech, H. J. Hansen, J. Jakobsen, and S. Haahr, "Presence of Epstein-Barr virus and human herpesvirus 6B DNA in multiple sclerosis patients: associations with disease activity," *Acta Neurologica Scandinavica*, vol. 112, no. 6, pp. 395–402, 2005.

[145] J. M. van Noort, J. J. Bajramovic, A. C. Plomp, and M. J. B. van Stipdonk, "Mistaken self, a novel model that links microbial infections with myelin-directed autoimmunity in multiple sclerosis," *Journal of Neuroimmunology*, vol. 105, no. 1, pp. 46–57, 2000.

[146] S. Cepok, D. Zhou, R. Srivastava, et al., "Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis," *Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1352–1360, 2005.

[147] A. Dolei, "MSRV/HERV-W/syncytin and its linkage to multiple sclerosis: the usability and the hazard of a human endogenous retrovirus," *Journal of NeuroVirology*, vol. 11, no. 2, pp. 232–235, 2005.

[148] A. Fryden, E. Kihlstrom, R. Maller, K. Persson, V. Romanus, and S. Ansehn, "A clinical and epidemiological study of "ornithosis" caused by *Chlamydia* psittaci and *Chlamydia pneumoniae* (strain TWAR)," *Scandinavian Journal of Infectious Diseases*, vol. 21, no. 6, pp. 681–691, 1989.

[149] S. Haidl, S. Ivarsson, I. Bjerre, and K. Persson, "Guillain-Barré syndrome after *Chlamydia pneumoniae* infection," *The New England Journal of Medicine*, vol. 326, no. 8, pp. 576–577, 1992.

[150] D. Michel, J. C. Antoine, B. Pozzetto, O. G. Gaudin, and F. Lucht, "Lumbosacral meningoradiculitis associated with *Chlamydia pneumoniae* infection," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 55, no. 6, p. 511, 1992.

[151] B. Sundelöf, H. Gnarpe, and J. Gnarpe, "An unusual manifestation of *Chlamydia pneumoniae* infection: meningitis, hepatitis, iritis and atypical erythema nodosum," *Scandinavian Journal of Infectious Diseases*, vol. 25, no. 2, pp. 259–261, 1993.

[152] M. Socan, B. Beovic, and D. Kese, "*Chlamydia* pneumonia and meningoencephalitis," *The New England Journal of Medicine*, vol. 331, no. 6, p. 406, 1994.

[153] M. Koskiniemi, M. Korppi, K. Mustonen, et al., "Epidemiology of encephalitis in children. A prospective multicentre study," *European Journal of Pediatrics*, vol. 156, no. 7, pp. 541–545, 1997.

[154] T. M. Korman, J. D. Turnidge, and M. L. Grayson, "Neurological complications of chlamydial infections: case report and review," *Clinical Infectious Diseases*, vol. 25, no. 4, pp. 847–851, 1997.

[155] A. Heick and E. Skriver, "*Chlamydia pneumoniae*-associated ADEM," *European Journal of Neurology*, vol. 7, no. 4, pp. 435–438, 2000.

[156] J. Guglielminotti, N. Lellouche, E. Maury, M. Alzieu, B. Guidet, and G. Offenstadt, "Severe meningoencephalitis: an unusual manifestation of *Chlamydia pneumoniae* infection," *Clinical Infectious Diseases*, vol. 30, no. 1, pp. 209–210, 2000.

[157] E. Anton, A. Otegui, and A. Alonso, "Meningoencephalitis and *Chlamydia pneumoniae* infection," *European Journal of Neurology*, vol. 7, no. 5, p. 586, 2000.

[158] L. Airas, P. Kotilainen, R. Vainionpää, and R. J. Marttila, "Encephalitis associated with *Chlamydia pneumoniae*," *Neurology*, vol. 56, no. 12, pp. 1778–1779, 2001.

[159] C. Boschin-Crinquette, A. Kreisler, L. Legout, P. Charpentier, A. Destée, and L. Defebvre, "Can meningoencephalitis be caused by *Chlamydiae pneumoniae*?" *Revue Neurologique*, vol. 161, no. 10, pp. 979–983, 2005.

[160] L. Wing, S. R. Leekam, S. J. Libby, J. Gould, and M. Larcombe, "The diagnostic interview for social and communication disorders: background, inter-rater reliability and clinical use," *Journal of Child Psychology and Psychiatry and Allied Disciplines*, vol. 43, no. 3, pp. 307–325, 2002.

[161] S. E. Folstein and B. Rosen-Sheidley, "Genetics of autism: complex aetiology for a heterogeneous disorder," *Nature Reviews Genetics*, vol. 2, no. 12, pp. 943–955, 2001.

[162] J. Veenstra-vanderweele, E. H. Cook Jr., and P. J. Lombroso, "Genetics of childhood disorders: XLVI. Autism, part 5: genetics of autism," *Journal of the American Academy of Child and Adolescent Psychiatry*, vol. 42, no. 1, pp. 116–118, 2003.

[163] H. Takahashi, S. Arai, K. Tanaka-Taya, and N. Okabe, "Autism and infection/immunization episodes in Japan," *Japanese Journal of Infectious Diseases*, vol. 54, no. 2, pp. 78–79, 2001.

[164] Y. Yamashita, C. Fujimoto, E. Nakajima, T. Isagai, and T. Matsuishi, "Possible association between congenital cytomegalovirus infection and autistic disorder," *Journal of Autism and Developmental Disorders*, vol. 33, no. 4, pp. 455–459, 2003.

[165] J. E. Libbey, T. L. Sweeten, W. M. McMahon, and R. S. Fujinami, "Autistic disorder and viral infections," *Journal of NeuroVirology*, vol. 11, no. 1, pp. 1–10, 2005.

[166] D. H. Thornton, "A survey of *mycoplasma* detection in veterinary vaccines," *Vaccine*, vol. 4, no. 4, pp. 237–240, 1986.

[167] J. K. S. Chia and L. Y. Chia, "Chronic *Chlamydia pneumoniae* infection: a treatable cause of chronic fatigue syndrome," *Clinical Infectious Diseases*, vol. 29, no. 2, pp. 452–453, 1999.

[168] R. Nicolson and P. Szatmari, "Genetic and neurodevelopmental influences in autistic disorder," *Canadian Journal of Psychiatry*, vol. 48, no. 8, pp. 526–537, 2003.

[169] W. Huang, D. See, and J. Tiles, "The prevalence of *Mycoplasma incognitus* in the peripheral blood mononuclear cells of normal controls or patients with AIDS or chronic fatigue syndrome," *Journal of Clinical Microbiology*, vol. 231, pp. 457–467, 1998.

[170] J. Nijs, G. L. Nicolson, P. De Becker, D. Coomans, and K. De Meirleir, "High prevalence of *Mycoplasma* infections among European chronic fatigue syndrome patients. Examination of four *Mycoplasma* species in blood of chronic fatigue syndrome patients," *FEMS Immunology and Medical Microbiology*, vol. 34, no. 3, pp. 209–214, 2002.

[171] G. L. Nicolson, R. Gan, and J. Haier, "Multiple co-infections (*Mycoplasma*, *Chlamydia*, human herpes virus-6) in blood of chronic fatigue syndrome patients: association with signs and symptoms," *APMIS: Acta Pathologica, Microbiologica et Immunologica Scandinavica*, vol. 111, no. 5, pp. 557–566, 2003.

[172] G. L. Nicolson, "Chronic bacterial and viral infections in neurodegenerative and neurobehavioral diseases," *Laboratory Medicine*, vol. 39, no. 5, pp. 291–299, 2008.

[173] G. Campadelli-Fiume, P. Mirandola, and L. Menotti, "Human herpesvirus 6: an emerging pathogen," *Emerging Infectious Diseases*, vol. 5, no. 3, pp. 353–366, 1999.

[174] D. Keen and S. Ward, "Austistic spectrum disorder: a child population profile," *Autism*, vol. 8, no. 1, pp. 39–48, 2004.

[175] B. Fellerhoff, B. Laumbacher, and R. Wank, "High risk of schizophrenia and other mental disorders associated with chlamydial infections: hypothesis to combine drug treatment and adoptive immunotherapy," *Medical Hypotheses*, vol. 65, no. 2, pp. 243–252, 2005.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 632513, 22 pages
doi:10.1155/2010/632513

*Review Article*

# Rhombencephalitis Caused by *Listeria monocytogenes* in Humans and Ruminants: A Zoonosis on the Rise?

**Anna Oevermann,[1] Andreas Zurbriggen,[1] and Marc Vandevelde[2]**

[1] *Neurocenter, Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, 3001 Bern, Switzerland*
[2] *Division of Clinical Neurology, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, 3001 Bern, Switzerland*

Correspondence should be addressed to Anna Oevermann, anna.oevermann@itn.unibe.ch

Received 4 August 2009; Accepted 25 November 2009

Academic Editor: Guey Chuen Perng



Listeriosis is an emerging zoonotic infection of humans and ruminants worldwide caused by *Listeria monocytogenes* (LM). In both host species, CNS disease accounts for the high mortality associated with listeriosis and includes rhombencephalitis, whose neuropathology is strikingly similar in humans and ruminants. This review discusses the current knowledge about listeric encephalitis, and involved host and bacterial factors. There is an urgent need to study the molecular mechanisms of neuropathogenesis, which are poorly understood. Such studies will provide a basis for the development of new therapeutic strategies that aim to prevent LM from invading the brain and spread within the CNS.

## 1. Introduction

The Gram-positive bacterium *Listeria monocytogenes* (LM) was first isolated in a human patient with meningitis 1921 and subsequently worldwide from a wide range of mammalian and nonmammalian species, notably farm ruminants [1–4]. However, it was not until the 1980s as a result of several human epidemics that listeriosis was recognized as a serious and frequently fatal foodborne disease and research activity on the disease was substantially intensified [5–7]. Since then the incidence has risen steadily including large outbreaks making listeriosis to a major public health issue [8–12]. Clinical syndromes associated with LM infection are similar in all susceptible hosts and include febrile gastroenteritis, septicemia, abortion, and central nervous system (CNS) infections such as meningitis, meningoencephalitis, and rhombencephalitis [11, 13, 14]. CNS involvement is a characteristic feature and accounts for the high mortality associated with listeriosis [11, 15, 16]. Currently, the agent is one of the best-studied bacterial pathogens for various reasons. Most importantly, it serves as model system for the study of innate and cell-mediated immunity, host-pathogen interactions, and intracellular survival of pathogens [17–25]. More recently, the bacterium has been investigated as a vector of heterologous proteins for vaccination and immunotherapy of cancer and infectious diseases [26–30]. However, although much progress has been made in these various fields of research, the pathogenesis and transmission of the CNS infection in its natural hosts, most notably the pathologically intriguing rhombencephalitis, is largely unknown. Particularly, not much is known about bacterial determinants that are associated with neurovirulence [31–33]. The purpose of this review is to summarize the current knowledge of the CNS form of LM infection in the natural host.

## 2. *Listeria monocytogenes*: An Emerging Foodborne Pathogen

LM belongs to the bacterial genus *Listeria*, which are Gram-positive, nonspore-forming, facultatively anaerobic, and intracellular coccobacilli. The genus comprises currently six

species including LM, *L. ivanovii, L. welschimeri, L. seeligeri, L. grayi*, and *L. innocua* [6]. Of those, only two species are considered potentially pathogenic: LM and *L. ivanovii* [6]. LM is the major pathogen of listeriosis and the only species of the genus that poses a serious public health risk. It causes invasive and often fatal disease including CNS infection in numerous animal species including farm ruminants, horses, dogs, pigs, deer, South American camelids, cats, and men. In contrast, *L. ivanovii* is considered only mildly pathogenic and seems to affect almost exclusively ruminants, causing abortion, still-births, and neonatal septicemia, but not CNS infections [4, 6, 34]. Both LM and *L. ivanovii* hold a group of virulence genes such as the positive regulatory factor A, internalins, hemolysins, phospholipases, a hexose phosphate transporter and others, which enable them to replicate within and spread between eukaryotic cells [21, 35, 36]. These virulence genes are absent or present in a nonfunctional form in the other four *Listeria* species that are considered primarily apathogenic saprophytes, although they have been very rarely isolated from humans and animals [37–43]. Accordingly, this review is confined to the discussion of LM.

LM is ubiquitously distributed and grows in a wide variety of environments including soil, water, plant matter, diverse food items, and intestinal tract of mammalian hosts [6]. In addition, the bacterium has well adapted to an intracellular life-cycle that is critical for its pathogenic potential. LM is a biofilm-producer and as compared to most other pathogenic bacteria relatively resistant to hostile environmental conditions including low pH, high salt concentrations and low temperatures [3, 44–46]. These properties render LM remarkably tenacious against numerous food-processing and food-preserving procedures and thus hazardous for the food industry. Hence, the bacterium has emerged as an important foodborne pathogen and is a major cause for large food recalls due to bacterial contamination [47, 48]. Although LM is able to infect a wide range of animal species, it occurs primarily in farm ruminants and humans [4, 14]. In both hosts, the prevalence of listeriosis has risen significantly since the 1980s resulting in intensified surveillance and control of LM in food industry, which contributed to a decrease of human listeriosis cases in the last two decades [49, 50]. However, in various European countries its prevalence has again increased in the last few years [9, 10, 51–53].

## 3. Listeriosis in Humans

*3.1. Incidence.* LM has been linked to sporadic episodes as well as large outbreaks of human illness worldwide [7–10, 12, 54–56]. The vast majority of human listeriosis cases occurs following consumption of contaminated food [57]. Although relatively rare (the annual incidence rate ranges from 1 to 10 cases per million), listeriosis has an important impact on public health given that it is responsible for the highest hospitalisation and mortality rates amongst foodborne infections and LM is a common food contaminant [15, 57, 58]. The case fatality rate ranges from 24% to 52% despite adequate antimicrobial treatment [11, 15, 59–65].

*3.2. Clinical Aspects.* LM has the propensity to cause invasive disease in well-defined risk groups including pregnant women, individuals at the extremes of age (newborns or elderly people), and patients with underlying conditions. The list of such underlying conditions is long and includes malignancies, diabetes mellitus, alcoholism, chronic hepatic and renal diseases, organ transplantation, autoimmune diseases, AIDS, immunosuppressive treatments (e.g., steroids), and treatments reducing the gastric acid secretion [6, 11, 14, 15, 52, 59–62]. However, listeriosis can occur in otherwise healthy individuals [8, 11].

The infection manifests in various syndromes, ranging from mild febrile gastroenteritis to serious invasive disease including septicaemia, abortions, and CNS disease [5, 6, 11]. In addition to these syndromes, listeriosis may present as a local infection including dermatitis, endocarditis, pericarditis, pneumonia, peritonitis, arthritis, hepatitis, and endophthalmitis [66–72]. Infection of nonpregnant adults leads to bacteremia and CNS disease in most cases [11, 13, 65], and listeriosis is nowadays the second to fifth most common etiology of human bacterial meningitis in the Western hemisphere [15, 59–63, 73–79]. The CNS form in humans generally develops as a diffuse meningitis/meningoencephalitis, usually associated with bacteremia. Meningitis prevails in neonates, elderly people and patients with immunosuppressive disorders or other concurrent conditions [11, 59, 60, 62, 74, 75, 80]. Less common CNS manifestations include abscesses in the cerebrum or cerebellum, and in up to 24% of patients encephalitis targeting the brainstem (rhombencephalitis), but the latter is probably under-recognized [13, 80–83]. Rhombencephalitis has been first described in 1957 by Eck as an unusual form of listeriosis [84]. In contrast to meningitis, it appears to occur predominantly in previously healthy patients without any predisposing conditions [13, 16, 82, 85]. The clinical course is usually biphasic, with a prodrome of unspecific symptoms consisting of headache, malaise, nausea, vomiting, and fever in the first phase during between 4 and 10 days, followed by progressive brainstem deficits with asymmetric cranial nerves palsy, cerebellar dysfunctions, hemi- or tetraparesis, sensory deficits, respiratory insufficiency, impairment of consciousness, and sometimes seizures [13, 81, 82, 85]. Blood and spinal fluid cultures are positive in 60% and 40% of patients, respectively [81, 82]. The condition is fatal unless treated early and survivors commonly have significant neurological sequelae [14, 81].

*3.3. Neuropathology.* In most cases of human listeriosis, the CNS form manifests as a diffuse suppurative meningitis occasionally also extending into the ventricles [86]. Rhombencephalitis involves primarily the medulla oblongata, pons and midbrain with infiltrates targeting frequently nuclei and tracts of cranial nerves [85, 87]. Lesions may extend into the cerebellum and further rostrally into the thalamus and basal nuclei [87]. Cellular infiltrations consist of agglomerates of microglial cells, microabscesses with neutrophils and macrophages, occasionally accompanied by neuronal necrosis and neuronophagia [87].

## 4. Listeriosis in Ruminants

*4.1. Incidence.* Listeriosis is of major veterinary importance in the three farm ruminant species cattle, sheep, and goats [4], not only by virtue of significant economical losses in livestock production due to morbidity and high mortality in animals, but also with regard to food safety and public health representing a possible link between the environment and human infection.

In ruminants, the foodborne route of LM infection has been well established long before it was shown in humans [4]. Many studies have indicated that poor-quality silage is commonly contaminated with LM and focused on spoiled silage as source for listeriosis outbreaks [4, 88–105]. In line with these results, fecal shedding of LM in cattle is associated with contamination of silage [106, 107]. The investigation of an epidemiological link between silage feeding and listeriosis in ruminants, however, gave inconsistent results. Whilst some studies could isolate matching LM strains in brains of affected animals and silage samples, others yielded unrelated strains [92, 94, 95, 98, 101, 108, 109]. A recent study detected a higher prevalence of the bacterium in samples collected from the immediate cattle environment (feed bunks, water through and beddings) and in cattle feces than in silage challenging the view that silage is the only source of LM infection [92]. This finding is in line with reports and our own observations of outbreaks unrelated to silage feeding [110–113].

Recent prevalence estimates of listeric encephalitis in cattle, sheep, and goats, based on neuropathological survey studies in Europe, range between 7.5% and 29.4% and a neuropathological survey of fallen stock in Switzerland identified listeriosis as the most important CNS disease of small ruminants [50, 114–117]. With reference to the small ruminant population in Switzerland the prevalence of listeric encephalitis was 216 cases/million sheep and 500 cases/million goats per year and thus exceeded significantly the number of human cases (between 1.4 and 9 cases/million inhabitants per year) [50, 58]. Similar data are not available for bovines. However, in neuropathological surveillance schemes for bovine spongiform encephalopathy in various countries, listeriosis scores as the most frequent neurological disease in cattle [114–117]. The importance of these data is underlined by significant economical losses in life stock industry caused by listeriosis, the likely role of ruminants as reservoir for human pathogenic strains and therefore its impact on food safety [118–121].

*4.2. Clinical Aspects.* The infection usually occurs in five distinct clinical presentations, of which encephalitis is by far the most common form, followed by abortions, whilst neonatal septicaemia, mastitis, and keratoconjunctivitis/uveitis occur quite rarely [3, 4, 102, 122]. These syndromes seldom overlap within the same animal or the same flock [4, 102, 123–127]. Some authors speculate that encephalitis occurs as a distinct syndrome and more frequently than other clinical syndromes in farm ruminants because immunity acquired through ingestion of contaminated silage protects against septicemia and abortion but is not fully effective in protection against encephalitis [4]. Furthermore, ruminants may commonly be asymptomatic intestinal carriers of the organism [90, 92, 128–130]. In contrast to humans, the classical CNS presentation in ruminants is rhombencephalitis, whereas diffuse meningitis or meningoencephalitis has only exceptionally been reported [131]. Listeric rhombencephalitis was first described in sheep as "circling disease" in New Zealand and since then has been reported in all three ruminant species around the world [4, 50, 98, 102, 105, 108, 111, 114, 125, 131–141]. Cattle appear to be less susceptible to the infection than small ruminants [4]. Occasionally, listeriosis may occur as an outbreak, particularly in sheep and goats [101, 104, 111, 113, 125, 127] (and own observations). The mortality rate is high despite antibiotic treatment [102]. Ruminants and in particular bovines are frequently exposed to relatively high environmental levels of LM cells [92, 120]. As in humans, the disease usually has a low attack rate affecting individual animals within a flock, although it is assumed that all animals are exposed to a similar infectious dose of LM [4, 102, 142, 143]. Therefore, most authors speculate that—similar to the situation in humans—hitherto unidentified underlying predisposing conditions facilitate the development of clinical listeric disease. In agreement with this hypothesis, cattle shed increased numbers of LM in their feces after transport stress [106]. However, although some authors described various concurrent conditions associated with natural listeriosis, the existence of such predisposing factors for listeric encephalitis has not yet been sufficiently proven neither in epidemiological investigations nor in experimental settings [4, 102, 111, 133, 144–155]. Furthermore, routine pathological examinations of ruminants with listeric rhombencephalitis uncommonly reveal significant concurrent disease (own observations). In this context, it is intriguing that the majority of human rhombencephalitis cases occurs in otherwise healthy individuals [82, 85, 156].

For unkown reasons, the incubation period for encephalitis is longer compared to the other conditions (septicaemia, abortion) and varies between 1 and 7 weeks [4, 105, 157–159]. Clinical signs of listeric encephalitis are similar in all three farm ruminants but vary depending on the topography of the CNS lesions. Generally they are characterized by unilateral or bilateral brainstem and cranial nerve (CN) deficits [4, 148, 160–162]. Common manifestations include masticatory problems, failure of jaw closure, hypoalgesia of the head (involvement of CN V), drooping of ears, upper eye lids and lips (involvement of CN VII), deficits of the palpebral and menace reflex (CNs V and VII), problems of swallowing (CNs IX and X), tongue palsy (CN XII), circling, head tilt and leaning to one side (vestibular system), nystagmus (CN VIII), and drooling of saliva (Figures 1 and 2). Other more unspecific signs include fever, dullness, and anorexia. In the terminal stage, animals become recumbent and may show convulsions. Rare cases have been described, in which limb paralysis occurred due to affection of the spinal cord alone (myelitis) without involvement of the brain [134, 163, 164]. The course of infection in small ruminants (sheep and goats) is generally acute and animals die within 1–3 days after clinical signs became apparent. In cattle, the course is more prolonged [4, 158].

Figure 1: Cow with rhombencephalitis due to *Listeria monocytogenes* infection. The cow has problems with swallowing (note the feed below her head) and shows increased salivation, facial paralysis with drooping of left ear and upper eyelid, courtesy of Dr. Mireille Meylan, Vetsuisse Faculty, University of Bern.

Figure 2: Recumbent goat with rhombencephalitis due to *Listeria monocytogenes* infection. The animal has a head-tilt and pleurothotonus; its right ear is drooping.

*4.3. Neuropathology.* Gross lesions of the brain are generally absent, but occasionally a greyish-tan discoloration and malacia or simply hyperaemic vessels can be observed in the brainstem. In contrast, histological lesions are pathognomic for the disease and include a combination of suppurative parenchymal lesions (microabscesses) and necrosis with perivascular lymphohistiocytic cuffings and gliosis (Figures 3 and 4). In severe cases, microabscesses may coalesce to large areas of suppuration. These changes are frequently accompanied by a meningitis. Lesions are commonly unilaterally pronounced and centered on the medulla oblongata and pons (Figure 3). However, they consistently spread rostrally into the midbrain, diencephalons, and telencephalon and caudally into the spinal cord [165]. Lesions in the rostral brain show a consistent topography in selective fiber tracts. The inflammatory process involves the ependyma and choroid plexi only in exceptional cases and is then always associated with rhombencephalitis [131, 165, 166].

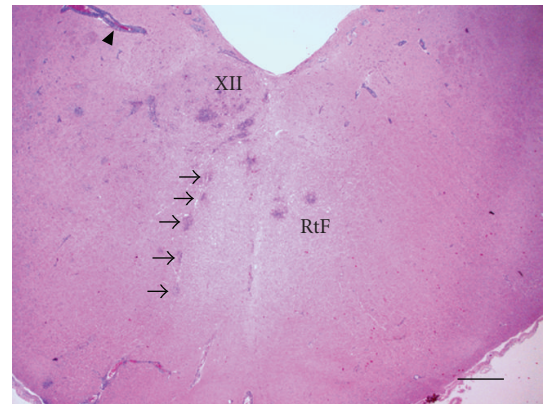



Figure 3: Rhombencephalitis in a sheep: Brainstem at the obex region with multiple microabscesses and perivascular cuffing (arrowhead). Microabscesses involve the hypoglossal nucleus (XII), its intracerebral root (arrows), and the reticular formation (RtF). Hematoxylin & Eosin stain (H&E), bar = 740 $\mu$m.

## 5. Pathogenesis of Listeriosis and Key Virulence Factors of *L. monocytogenes*

The pathogenicity of LM depends on its capacity to resist hostile environmental conditions and invade and replicate in both professional phagocytes and nonphagocytic host cells, which is determined by at least 50 genes scattered in the genome [22, 167]. Our knowledge of the pathogenesis at the host and cellular level largely derives from infections in various laboratory animals, notably the mouse, and *in vitro* models.

*5.1. Cellular Interactions.* At the cellular level, the infection cycle is regulated by the synchronized operation of various virulence factors. The intracellular life cycle and the intercellular spread of LM has been intensively studied revealing its molecular adaptation to the intracellular microenvironment. The reader is, therefore, referred to various reviews for

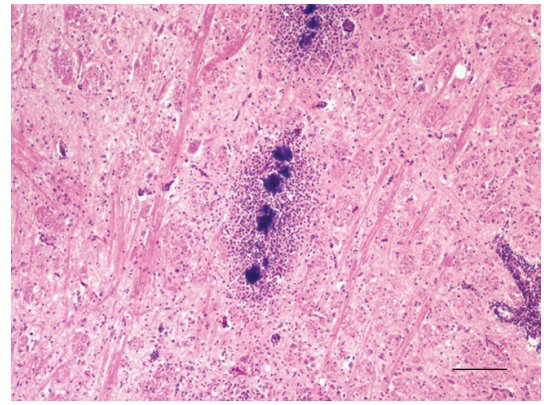


Figure 4: Rhombencephalitis in a sheep: Microabscesses with central bacterial colonies aligned along an axonal tract within the reticular formation (medulla oblongata). H&E, bar = 100 $\mu$m.

detailed information [17–19, 21, 168]. The invasion of the host cell is mediated by interaction between internalins, listeric surface ligands, and their respective host cell-receptors. A large number of internalins and internalin-like proteins have been identified by genome sequencing analysis of several LM strains [18, 169–171] and the diversity of internalins within the range of the different *Listeria* species and LM strains could possibly explain the variation in virulence and pathogenicity [172]. Amongst those, Internalin A and B (InlA, InlB) are the best studied and these have been detected only in LM so far [6, 173, 174]. The former interacts with E-cadherin, which is mostly expressed on epithelial cells in species-specific manner [175]. Notably, E-cadherin is expressed on cells of three host barriers that could determine the clinical syndromes: intestinal barrier, blood-brain barrier, and placental barrier [176–179]. In contrast, InlB promotes the invasion of a wide variety of mammalian cells through interaction with three receptors: Met, globular C1q receptor (gC1qR), and proteoglycans [180–183]. Recently, other internalins, notably internalin J, have been identified as key factors for virulence of LM [184–190]. There is recent evidence that entry of LM into the host cell requires additional factors such as clathrin-mediated endocytosis [23, 191, 192]. Once within the cell, LM is caught in a single-layer membrane phagocytic vacuole and has to transiently resist phagosomal killing [193, 194]. The bacterium escapes from the phagosome and moves into the host cell cytoplasm by employing a pore-forming toxin, listeriolysin-O (LLO), assisted by two phospholipases, phosphatidyl-inositol phospholipase C (PlcA) and phosphatidylcholine phospholipase C (PlcB) [195, 196]. Free in the cytoplasm, LM replicates rapidly [197, 198]. A surface protein of LM, Actin A (ActA), recruits host actin filaments and induces their polymerization to a so-called actin comet tail at one bacterial pole enabling the bacterium to move freely within the cytoplasm and to spread to neighboring cells by formation of cellular membrane protrusions that are engulfed by adjacent cells [17]. The resulting secondary double-membrane vacuole within the neighbor cell is lysed by PlcB and LLO and a new infection cycle starts over again [199]. It is believed that the direct intercellular spread permits the bacterium to multiply and diffuse within tissues protected from host defenses by avoiding the contact with the extracellular compartement.

*5.2. Infectious Process In Vivo.* Whilst the different steps of the intracellular infection cycle and key virulence factors involved are well known [6, 18, 19, 21, 200], the knowledge of the infectious process *in vivo*, notably in the natural host, is currently limited. It is generally believed that LM enters the host primarily through the intestine after oral intake of contaminated food. In rare cases, direct skin exposure to LM, for example, through contaminated abortive material, may lead to cutaneous infections [201, 202]. Several virulence factors enable LM to resist the exposure to a highly acidic environment, proteolytic enzymes, and bile salts during its gastroduodenal passage [44, 203–209]. Subsequently, LM crosses the intestinal barrier by actively adhering to and invading enterocytes through interactions between host-cell receptors and internalins, namely, InlA [178]. After intestinal translocation it invades the bloodstream and reaches liver and spleen (primary target organs) hematogeneously. There, resident hepatic and splenic macrophages kill the invading bacteria leading to control of the infection [210]. This initial step is thought to be subclinical and common due to the high prevalence of LM in food. In normal individuals, such exposure to listerial antigens probably contributes to the maintenance of memorial T-cells [211]. The unrestricted replication of LM in the primary target organs as it may occur in immunocompromized individuals may result in hematogenous dissemination to other organs and in overt clinical disease. LM has a predilection for the placenta and CNS (secondary target organs) that determines the main clinical syndromes. This predilection is believed to reflect the inherent ability of LM to cross the blood-brain and the placental barrier, likely by the interaction of bacterial internalins and their host cellular receptors [17, 21]. Recently, it has been shown that both InlA and B are required for the crossing of the placenta [177, 212]. In contrast, such an interaction remains to be shown to mediate the crossing of the blood-brain barrier.

Much of the information reviewed above has been derived from experimental work in laboratory rodents and cell cultures, whereas the role of LM virulence factors in its natural hosts is practically not known. InlA may play a key-role in human virulence as it is indicated by an epidemiological study, which detected a truncated form of InlA in 35% of LM food isolates versus only 4% of clinical isolates [213].

*5.3. Genomic Organisation of Virulence Genes.* The whole-genome sequences of LM and of the related nonpathogenic *Listeria innocua* and *Listeria welshimeri* have been determined, and their comparison has pioneered the identification of virulence factors of LM [169, 170, 214–216]. Two clusters of genes are required for the intracellular life-cycle of LM. The genes that encode the key virulence factors PlcA, LLO, ActA, and PlcB are clustered in a 10 kb virulence locus on the chromosome, the Listeria pathogenicity island 1 (LIPI-1), and are under the control of a transcriptional activator, the positive regulatory factor A (PrfA), [200, 217]. The latter itself is regulated by environmental conditions, namely, the temperature [218–220]. At mammalian host temperature (37°C), PrfA is translated and thus PrfA-dependent virulence genes are transcribed permitting LM to switch from an environmental bacterium into a intracellular pathogen. The second cluster consists of an operon with only two genes, *inlA* and *inlB*. Additionally to *inlA* and *inlB*, a high number of virulence genes encoding for internalin-like genes are scattered within the genome [171].

## 6. Neuropathogenesis

The means by which LM invades the brain have been subject of speculation for decades in both human and veterinary medicine [4, 14, 221]. From the pathological point of view,

the variation of neuropathological patterns that are associated with CNS infection suggests strongly that the pathogen is able to invade the brain by both hematogenous spread or by migration along axons. However, the pathogenesis of both major manifestations of CNS infection—meningitis and rhombencephalitis—is largely unknown. Notably, the infectious dose required host and pathogen factors involved, particularly the role of LM virulence factors, reasons for the low attack rate, molecular mechanisms of brain invasion, and dynamics of the CNS infection including factors determining the outcome remain challenging.

*6.1. Meningitis.* The meningeal form with its diffuse distribution—as it occurs frequently in humans—is likely to be a result of hematogenous spread to the brain and crossing of the blood brain barrier. Indeed, in murine models of listeriosis bacteraemia is required for CNS invasion and lesions as well as bacteria are mainly observed in the meninges, choroid plexi and ependyme [222–225]. *In vitro* and *in vivo* experiments could show that LM is able to cross the blood-brain barrier by direct invasion of endothelial cells, cell-to-cell spread from infected phagocytes to endothelial cells, or by entry between endothelial cells within infected phagocytes [222, 226–232]. At present, the molecular mechanisms of breaching the blood-brain barrier by LM are still virtually unknown. Given that the endothelium and the choroid plexus epithelium of the blood-brain barrier may express E-cadherin [179, 233–235], some authors suggest that a receptor interaction between the cellular E-cadherin and bacterial internalin A might be the underlying mechanism of blood-brain-barrier crossing, similar to what happens at the intestinal and placental barrier [17, 21, 177, 212]. Epidemiologic data in humans do not indicate that InlA contributes to neuroinvasion from the bloodstream [213]. Two further virulence genes have been proposed to play a role in CNS infection based on investigations of mutant strains in the mouse model, plcB and gtcA, a gene that mediates teichoic acid glycolisation. PlcB is not indispensable, since plcB-negative mutants were able to cause delayed encephalitis in the mouse-model [31]. Mutations involving the gtcA gene that encodes putative cell wall components caused attenuated growth of LM in the brain of mice, and thus the authors speculate that these mutations caused a lower efficiency in the passage of the blood-brain-barrier [32].

*6.2. Rhombencephalitis in the Natural Host.* In humans, rhombencephalitis occurs in up to 24% of listeriosis patients [13, 80–83]. Both distribution and nature of the lesions are very similar in listeric rhombencephalitis of people and ruminants [87, 165]. However, despite the significant losses in livestock industry due to listeriosis and the growing impact of this zoonosis in ruminants and humans [8, 9, 50], surprisingly few studies have been focused on the pathogenesis of encephalitic disease in its natural hosts in the last decades [33, 165, 236]. This is in contrast to the large number of studies on listeric CNS disease in mice and rats, which are not naturally susceptible to LM infection due to species-specific properties of E-cadherin that functions as a receptor for internalin A [31, 175, 222–225, 237–252]. Because experimental data and observations in natural disease diverge, the pathogenesis of listeric rhombencephalitis and particularly the mechanisms of brainstem predilection are still controversial [131, 135, 136, 166, 222, 253]. However, the neuropathological pattern of the natural disease and the observation of intraaxonal and intraneuronal bacteria (Figures 5 and 6) strongly suggest that foodborne LM cells invade the brainstem by axonal migration along various cranial nerves [87, 131, 136, 165, 254]. Correspondingly, in an outbreak of listeric myelitis in sheep ascending infection via the sensory nerves following dermatitis was suspected and subcutaneous injection of LM in the lumbar and thoracic regions may cause lumbar and thoracic myelitis in mice [163, 255]. These observations indicate that the site of bacterial invasion determines the topography of CNS lesions. Once in the brainstem, LM likely spreads further rostrally to higher brain centers and caudally to the spinal cord along axonal connections [165]. According to this view, isolation of the agent from the cerebrospinal fluid (CSF) in ruminant encephalitis usually fails indicating that it rarely enters the CSF during infection [137]. The intriguing topography of lesions that hit systematically and particularly the rhombencephalon is atypical for a bacterium. As a general rule, bacteria invade the brain hematogenously. During septicemia, bacteria may cross the blood-brain-barrier at the level of the microvasculature causing meningitis, choroiditis, and ependymitis. Alternatively, microorganisms may travel to the brain lodging in septic thromboemboli that get trapped within parenchymal vessels and produce disseminated suppurative lesions within the brain parenchyma that with time develop to abscesses [86]. In this context, it is fascinating that a second ubiquitous and facultatively intracellular bacterium, which is able to spread from cell to cell by actin-polymerization—*Burkholderia pseudomallei*—causes a brainstem encephalitis in men and animals akin to that of LM [256–261]. Therefore, it is thought that *Burkholderia pseudomallei* like LM probably moves to the brainstem by centripetal axonal migration. Furthermore, in the vast majority of cases LM is isolated from the brain, but not from other organs [131, 158, 262]. Taken together, these data do not support a hematogenous infection. LM rather enters submucosal nerve endings through mucosal injuries anywhere in the oropharyngeal and nasal cavity, lips, conjunctiva, or gut [135, 165]. It has been attempted to infect sheep using various inoculation routes including intramuscular, intradermal, subcutaneous, intracarotid, intracerebral, intravenous, oral, intraruminal, intravaginal, and conjunctival inoculation [133, 145–147, 152, 262–265]. However, typical lesions of rhombencephalitis could only rarely be reproduced, and clinical responses after the subcutaneous injection of LM in sheep and goats or after oral challenge are generally minimal [3, 133, 263, 265, 266]. In contrast, experimental exposure of injured oral mucosa to LM and intranerval inoculations may cause lesions in the brainstem of mice, sheep and goats reminiscent of the natural disease [135, 157, 159, 237, 241, 267, 268]. Myelitis, and radiculitis in sheep

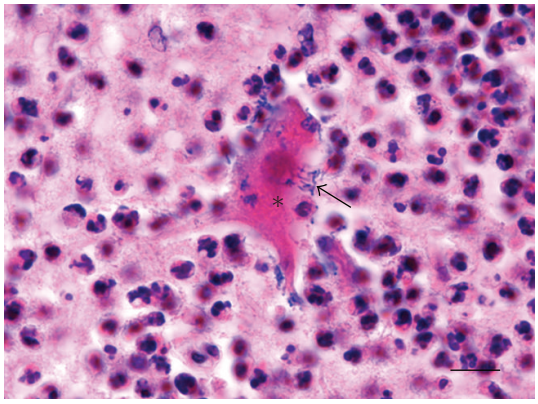


Figure 5: Rhombencephalitis in a sheep: necrotic neuron (asterisk) with intraneuronal *Listeria monocytogenes* (arrow) in a microabscess of the medulla oblongata. H&E, bar = 13 $\mu$m.

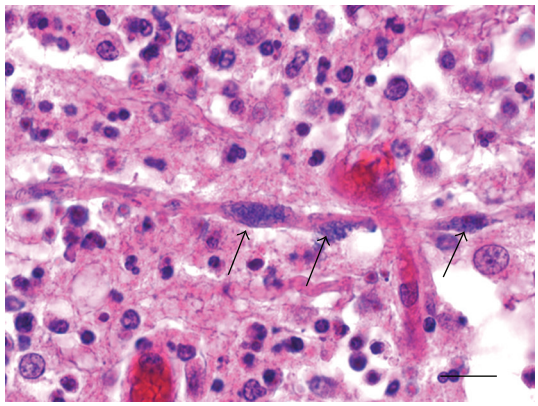


Figure 6: Rhombencephalitis in a sheep: intraaxonal *Listeria monocytogenes* (arrows) within a microabscess of the medulla oblongata. H&E, bar = 13 $\mu$m.

were produced by injecting LM into the left infraorbital nerve [269].

Some groups claim that hematogenous infection with LM leads to rhombencephalitis, and targeting of the brainstem is determined by its high microvascular density [166, 222, 244, 270]. However, lesions were frequently not restricted to rhombencephalitis, but included additional severe and diffuse choroiditis, meningitis and disseminated microabscesses in other brain areas, which is in contrast to the natural disease.

*6.3. Rhombencephalitis in Experimental Laboratory Animals.* Experimental research on listeric encephalitis, however, yielded contradictory results. The main obstacle for the study of listeric encephalitis is the lack of an animal or *in vitro* model that reflects the natural infection and consistently reproduces rhombencephalitis. Most experiments in laboratory rodents, notably the mouse, reflect unnatural infection routes by employing intracerebral or intravenous infection, as these species are highly resistant to lethal infection and CNS invasion following oral inoculations of LM, which imitate the natural route of infection, due to an amino acid mutation in E-cadherin, the cellular receptor for InlA [175]. Although an increasing number of studies aim towards natural routes of infection [176, 223], numerous studies still employ parenteral inoculations. This problem may be overcome in future with the use of the genetically engineered E16P knock-in mouse that expresses human E-cadherin in all tissues [271]. Animals inoculated intracerebrally or intravenously suffer a severe and diffuse meningoencephalitis and choroiditis, but not rhombencephalitis [31, 222–225, 245–247, 250, 251]. Differences in neuropathological expression of CNS listeriosis between laboratory animals and the natural host may not only reflect variation in exposure route but also species-specific anatomical and physiological differences of the brain. Furthermore, most experimental studies of encephalitis in laboratory animals and cell cultures have been carried out with the LM EGD strain and mutants, which are serotype 1/2a and may not reflect the virulence mechanisms of the other two clinically important serotypes 1/2b and notably 4b [31, 32, 222, 239, 241, 242, 270, 272]. Therefore, although laboratory animal models have contributed to the understanding of the pathogenesis in listeriosis, the results obtained in these models cannot be automatically extrapolated to humans and ruminants, since small rodents are not naturally susceptible to LM infection.

*6.4. Mechanisms of Neural Spread.* Although many data strongly indicate a local invasion via centripetal migration along axons, the mechanisms of this process are virtually unknown. The first riddle to solve is how LM is able to pass the mucosal barrier and enter the submucosal nerve endings. At present, it is thought that LM passes the mucosal epithelium of the upper gastrointestinal tract through small mucosal abrasions. There are two potential scenarios that may explain axonal invasion of cranial nerves: it may occur when LM surface proteins interact with a yet unidentified membrane receptor on the axonal surface or by cell-to-cell spread from infected macrophages in the submucosa [240]. The former hypothesis is supported by the apparently selective infection of neuronal populations *in vitro* [239]. Potential candidates would be InlA and InlB, which have both been shown to be required for the invasion of other cell types [176–178, 180–183, 212]. Interestingly, the cellular receptor of InlA, E-cadherin, is expressed in murine neuronal subpopulations such as sensory neurons of the trigeminal and dorsal root ganglion [273–275]. Most experimental data favor an axonal invasion via cell-to-cell spread from infected macrophages. In contrast to the observation of intraneuronal and intraaxonal LM during natural encephalitis, *in vitro* data of experimentally infected rat spinal and ovine brain cell cultures indicate that the bacterium rarely infects neurons [253, 276]. The infection rate increases when neurons are cocultivated with infected macrophages, indicating that LM infects neurons rather by cell-to-cell spread than by direct invasion through receptor interaction [276]. Further evidence for such a cell-to-cell spread of LM from macrophages comes from *in vivo* data in mice indicating that macrophages and dendritic cells facilitate neuroinvasion [272].

The affection of both motor and sensory nerves indicates that LM spreads by antero- and retrograde axonal migration to the neuronal bodies of brainstem and midbrain, likely by employing its actin tail as suggested by experimental data [87, 165, 239, 240]. Transganglionic migration within sensory nerves and further intracerebral spread between functionally connected neuronal cell populations likely occur via cell-to-cell spread. This would be in line with the importance of PlcB, a virulence factor promoting cell-to-cell spread, in the pathogenesis of experimental listeric meningoencephalitis [31].

It is important to note that much of the information reviewed above has been derived from experimental work in laboratory rodents and cell cultures. Thus, it remains to be demonstrated that these findings are applicable to the disease in its natural hosts.

## 7. Strain Variation in Relation to Neurovirulence in Humans and Ruminants

The species LM encompasses numerous strains and the genetic diversity amongst them is high [277]. Various strains have been implicated in both human and animal disease and it is not clear which LM subtypes in the environment cause illness. Thus, current surveillance schemes for foods are based on the assumption that all LM isolates are potentially pathogenic resulting in costly recalls in food industry. However, epidemiological studies conjoint with strain subtyping by diverse methods (such as serotyping or genomic approaches) suggest that there are, as yet, poorly understood interstrain differences in virulence and transmission [120, 278–283]. Therefore, research in recent years focused on the identification of molecular markers that determine the strain variation in virulence. Although progress has been made, the conundrum is only fragmentarily unraveled and with regard to the neurological disease it is virtually unknown whether neurotropic strains exist and what determines their propensity for neuroinvasion.

*7.1. Serotypes.* Although at least 13 serotypes are known, more than 95% of clinical isolates from human epidemics or sporadic cases belong to only three serotypes: 1/2a, 1/2b, and notably 4b, which are not the most common strains amongst environmental and food isolates [213, 279, 281, 284–287]. On the other hand, other serotypes are rarely responsible for human disease regardless of their common isolation from food or environmental specimens [277, 282, 288, 289]. However, food strain types and clinical strains partially overlap and key virulence genes are present in all serotypes [118, 290–293]. Food isolates, though, show more genetic diversity than clinical strains, suggesting that only certain food-derived strains may cause human infection [294]. The majority of LM strains that account for large but temporally and geographically unrelated outbreaks of food-borne listeriosis appear to form two epidemic clones in the serotype 4b, independently of the contaminated source involved [15, 279, 286, 295–297]. This serotype is also responsible for the majority of sporadic infections and is apparently overrepresented in pregnancy-associated cases and meningoencephalitis, whilst 1/2b has been primarily associated with nonpregnant individuals with severe underlying illness and HIV infections [279, 285, 298–301]. Taken together, these data suggest that serotype 4b is more virulent than other serotypes of LM. In line with these results, another study revealed a higher mortality rate in patients infected with strains of serogroup 4b as compared to other serotypes [302].

*7.2. Genotypes.* The application of genomic subtyping methods resulted in two major evolutionary lineages (lineages I and II) and one minor lineage (lineage III) of LM that apparently differ in host specifity and pathogenic potential [282, 283, 293, 297, 303–305]. Thereof lineage I is highly clonal and contains all 4b food-borne-epidemic isolates despite the different countries concerned and the food vehicles involved as well as additional isolates from sporadic cases. In contrast, lineage III contains no human clinical isolates. Lineage II shows a greater genetic diversity and contains clinical isolates but apparently no isolates from food-borne epidemics. One study that employed repetitive element sequence-based PCR could allocate food isolates in another genomic cluster than clinical isolates from human and animals [306].

*7.3. Correlation of LM Strains with Pathogenicity.* At present, it is not known though whether the observed divergence in subtype distribution between clinical and environmental isolates reflects potential variation in virulence or adaptation to particular ecological niches (e.g., food processing plants) enabling certain serotypes to contaminate food products at infectious levels [118]. One further explanation for the divergence would be a transmission route different than foodborne [289]. However, in either case a molecular basis for variations remains to be discovered. First steps have been done with the discovery of low-virulent strains that account for a significant proportion of environmental isolates [278, 307–312]. Mutations in their key virulence genes including *inlA*, *inlB*, *plcB*, *prfA*, *hly* (LLO-encoding gene), or *actA* have been detected [313–317]. However, the low virulence is mainly determined by point mutations in diverse virulence genes, which are impossible to detect by most subtyping methods [317]. Accordingly, attempts to use key virulence proteins and genes as targets for discrimination of virulent from avirulent LM strains generally failed because both proteins and genes were present in the entire strain population studied independently of their origin [282, 318–320]. An exception is the low virulence of some strains determined by a truncated form of InlA. An epidemiological study could show that the full-length form of InlA was expressed by 96% of clinical LM isolates versus 65% of food isolates [213, 313]. Internalin J (lmo 2821) is another virulence factor claimed to be a putative marker for differentiation between virulent and avirulent strains as it is invariably present in virulent strains [184, 185, 321].

As epidemiological data suggest interstrain variation in virulence, strong efforts have been made to develop *in vitro* tests and animal models that reflect the variation in virulence between clinical and environmental strains, though with inconsistent results. Although reproducible virulence differences were observed between LM strains in both cell cultures and animal models [278, 308–311, 322–326], not all studies found a correlation of the virulence in the laboratory with the serotype or the source (e.g., clinical isolate, food isolate) [309, 327]. However, several mouse studies observed a higher infectivity of serotypes 1/2a, 1/2b, 1/2c, and 4b strains than other serotypes [184, 328–330] and some *in vitro* studies observed virulence differences between clinical and food isolates [307, 326, 331], which is in line with the epidemiological observations.

*7.4. L. monocytogenes Strains in Farm Animals.* Although little is known about the distribution of clinical LM strains in animals, there is some epidemiological evidence that—like in humans—interstrain differences in virulence and organ tropism exist. In farm ruminants, the different clinical forms rarely overlap in the same herd, and visceral and cerebral listeriosis only exceptionally occur simultaneously in the same animal [126, 282, 332]. Furthermore, LM may cause encephalitis in pregnant ruminants without inducing abortion [262, 333] (own observations). However, the significance of divergent LM strains in the pathogenesis of ruminant listeriosis is not well known. As in humans, serovars 1/2a, 1/2b, and 4b appear to be the most commonly isolated LM strains in ruminants and ruminant LM isolates belong to all three identified evolutionary lineages [162, 282].

*7.5. Factors of Neurovirulence.* Whilst CNS infection is substantially responsible for the high mortality in both human and ruminant listeriosis, the identification of neurovirulence factors has not received much attention. The particular neuropathological pattern of rhombencephalitis and the absence of other organs involvement in previously healthy patients and ruminants suggests that LM strains with neurotropism exist. But with regard to molecular markers that determine strain variation in neuroinvasion and neurovirulence research is in the dark. A major handicap is the lack of an adequate animal or *in vitro* model to define and measure neurovirulence.

Older publications describe that in natural ruminant encephalitis either serotype 1 or 4b prevails depending on the geographical area [158, 334]. In experimental infection of sheep, one serotype 4 isolate showed high neurovirulence, whilst serotype 1 did not induce rhombencephalitis [157]. Similar results have been reported by other authors [267, 268, 335, 336]. In this context, it is worthy of note that occasionally different subtypes of LM may be isolated from clinically affected animals during an outbreak [101, 110, 334]. Interestingly, a particular phage type of LM was associated with an unusually high incidence of rhombencephalitis during a Swiss outbreak of human listeriosis [156].

PlcB has been proposed as a virulence factor for encephalitis, but PlcB is not indispensable, since PlcB-negative mutants were able to cause delayed encephalitis in the mouse-model [31]. An epidemiological study of human clinical isolates could associate CNS infections with two particular ActA subtypes [287]. Wiedmann identified one particular LM ribotype that was strongly associated with encephalitis in cattle indicating that this ribotype might be a host-associated subtype [282], and a recent study suggests that lineage I strains may have neurotropism in cattle [337]. These authors observed that encephalitic strains in cattle posses a specific internalin profile (lacking *inlF* and *inlG*) and a specific *actA* type (lacking one *actA* proline-rich repeat) and therefore speculate that gene-loss events and deletions may be associated with virulence and tissue specificity of the different strains. However, the importance of these genes in neurovirulence and neuroinvasion, whether by hematogenous infection or axonal migration, is not known.

## 8. Are Ruminants a Zoonotic Reservoir for Human Rhombencephalitis Strains?

The link between ruminant and human listeriosis is not completely understood. Listeriosis is defined a zoonosis, but direct transmission between ruminants and humans rarely occurs and is in most cases associated with nonlife-threatening cutaneous infections through contact with infected cattle or after handling of abortive material [201, 202]. However, it appears reasonable to implicate ruminants as an important natural reservoir for strains causing human infections given that one epidemic clone responsible for a significant proportion of human epidemics has been frequently isolated from cases of ruminant listeriosis [118, 121, 282, 297, 338]. Furthermore, dairy farms are frequently contaminated with LM, particularly as compared to other environments, and its subtype populations in the farm environment encompass commonly strains that have been associated with human illness, whether sporadic or epidemic [48, 90, 92, 107, 119, 120, 128, 139, 291, 339–345]. Ruminants, particularly cattle, contribute to amplification and dispersal of LM into the farm environment [120]. The bacteria can be shed in the feces of clinically affected animals, but also healthy carriers [90, 92, 107, 119, 128, 130, 339, 346, 347]. Raw milk might contain LM either as a consequence of bacterial shedding in the milk or due to exogenous contamination from the dairy farm environment [291, 340, 348–355].

Human listeriosis is principally a food-borne infection and most reported outbreaks of listeriosis in men are attributed to the consumption of contaminated products of animal origin [15, 58, 339, 340, 356–362]. Transmission may occur indirectly through food products from infected animals or healthy carriers that are not processed before consumption as well as raw vegetables that are contaminated by LM containing manure [363]. Most foods of animal origin are treated by procedures that effectively kill LM in raw foods. Therefore, a possible means of transmission of LM strains from ruminants to humans is their introduction

and establishment in food processing facilities, and their ability to produce biofilms and to adhere to inert surfaces may significantly contribute to the latter [46, 364, 365]. Supporting this hypothesis, one study identified several LM genotypes that contaminated both dairy-processing and farm environments [366].

Although all these results strongly implicate ruminants as a natural reservoir for LM and a source of human infections, at present, there are no data with regard to the extent of strain population overlap between human and ruminant rhombencephalitis. The identical neuropathology of listeric rhombencephalitis in humans and ruminants, however, indicates that neurotropic strains common to both hosts are responsible for the disease.

## 9. Conclusions

Rhombencephalitis is an apparently uncommon form of listeriosis in humans, but its prevalence is likely underestimated [13, 80–83]. In contrast, in ruminants it is the most common clinical expression of listeriosis and at the same time the most common CNS disorder [50]. The intriguing distribution and the nature of the lesions are very similar in listeric rhombencephalitis of people and ruminants [87, 165]. Furthermore, ruminants may shed high numbers of LM in their feces [90, 92, 107, 119, 128, 130, 339, 346, 347], and dairy farms are frequently contaminated [92, 120]. Until recently, it has been believed that all LM strains are potentially pathogenic. However, epidemiological evidence suggests that there are strain-specific variations in virulence and research has identified strain variation in respect to virulence in animal and cell culture models, although the results frequently do not correlate with epidemiological data. Taken together, these data indicate that neurotropic strains of LM common to both humans and ruminants might cause the rhombencephalitis and that ruminants and their close environment may be their natural reservoir. The identification of virulent strains causing rhombencephalitis and their differentiation from avirulent and low-virulent strains would help to implement effective control and prevention measures against LM. In the context of the reported increase of LM infections in humans [8–10] and the high prevalence of listeric rhombencephalitis in ruminants [50] there is an urgent need to study host and bacterial factors, which contribute to listeric rhombencephalitis, and notably the molecular mechanisms of neuroinvasion, which are poorly understood. Future research might focus on the identification of candidate bacterial proteins and the respective host cell receptors that determine host cell specificity and tissue tropism. The first steps have been done by identifying the key-players in the crossing of the intestinal and placental barrier. Now it is time to search for those that enable the agent to invade the brain.

## Acknowledgment

This work was financed by the Swiss Federal Veterinary Office.

## References

[1] J. Dumont and L. Cotoni, "Bacille semblable à celui du rouget du porc rencontré dans le L.C.R. d'un méningitique," *Annales de l'Institut Pasteur*, vol. 35, pp. 625–633, 1921.

[2] E. G. D. Murray, A. A. Webb, and M. B. R. Swan, "A disease of rabbits characterized by a large mononuclear monocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* n. sp.," *Journal of Pathology & Bacteriology*, vol. 29, pp. 407–439, 1926.

[3] M. L. Gray and A. H. Killinger, "*Listeria monocytogenes* and listeric infections," *Bacteriological Reviews*, vol. 30, no. 2, pp. 309–382, 1966.

[4] J. C. Low and W. Donachie, "A review of *Listeria monocytogenes* and listeriosis," *Veterinary Journal*, vol. 153, no. 1, pp. 9–29, 1997.

[5] W. F. Schlech III, P. M. Lavigne, R. A. Bortolussi, et al., "Epidemic listeriosis—evidence for transmission by food," *New England Journal of Medicine*, vol. 308, no. 4, pp. 203–206, 1983.

[6] J. A. Vázquez-Boland, M. Kuhn, P. Berche, et al., "*Listeria* pathogenesis and molecular virulence determinants," *Clinical Microbiology Reviews*, vol. 14, no. 3, pp. 584–640, 2001.

[7] M. J. Linnan, L. Mascola, X. D. Lou, et al., "Epidemic listeriosis associated with Mexican-style cheese," *New England Journal of Medicine*, vol. 319, no. 13, pp. 823–828, 1988.

[8] H. de Valk, C. Jacquet, V. Goulet, et al., "Surveillance of *Listeria* infections in Europe," *Euro Surveillance*, vol. 10, no. 10, pp. 251–255, 2005.

[9] V. Goulet, C. Hedberg, A. Le Monnier, and H. de Valk, "Increasing incidence of listeriosis in France and other European countries," *Emerging Infectious Diseases*, vol. 14, no. 5, pp. 734–740, 2008.

[10] J. Denny and J. McLauchlin, "Human *Listeria monocytogenes* infections in Europe—an opportunity for improved European surveillance," *Euro Surveillance*, vol. 13, no. 13, article 5, p. 8082, 2008.

[11] Y. Siegman-Igra, R. Levin, M. Weinberger, et al., "*Listeria monocytogenes* infection in Israel and review of cases worldwide," *Emerging Infectious Diseases*, vol. 8, no. 3, pp. 305–310, 2002.

[12] Public health agency of Canada, "*Listeria monocytogenes* outbreak," Anonymous, 2009, http://www.phac-aspc.gc.ca/alert-alerte/listeria/listeria_2009-eng.php.

[13] R. Bartt, "*Listeria* and atypical presentations of *Listeria* in the central nervous system," *Seminars in Neurology*, vol. 20, no. 3, pp. 361–373, 2000.

[14] D. A. Drevets and M. S. Bronze, "*Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion," *FEMS Immunology and Medical Microbiology*, vol. 53, no. 2, pp. 151–165, 2008.

[15] C. J. Büla, J. Bille, and M. P. Glauser, "An epidemic of foodborne listeriosis in western Switzerland: description of 57 cases involving adults," *Clinical Infectious Diseases*, vol. 20, no. 1, pp. 66–72, 1995.

[16] R. Malinverni, J. Bille, C. Perret, et al., "Epidemic listeriosis. Report of 25 cases in 15 months at the Vaud University Hospital Center," *Schweizerische Medizinische Wochenschrift*, vol. 115, no. 1, pp. 2–10, 1985.

[17] J. Pizarro-Cerda and P. Cossart, "Subversion of cellular functions by *Listeria monocytogenes*," *Journal of Pathology*, vol. 208, no. 2, pp. 215–223, 2006.

[18] M. Hamon, H. Bierne, and P. Cossart, "*Listeria monocytogenes*: a multifaceted model," *Nature Reviews Microbiology*, vol. 4, no. 6, pp. 423–434, 2006.

[19] S. Seveau, J. Pizarro-Cerda, and P. Cossart, "Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion," *Microbes and Infection*, vol. 9, no. 10, pp. 1167–1175, 2007.

[20] P. Cossart, "Listeriology (1926–2007): the rise of a model pathogen," *Microbes and Infection*, vol. 9, no. 10, pp. 1143–1146, 2007.

[21] P. Cossart and A. Toledo-Arana, "*Listeria monocytogenes*, a unique model in infection biology: an overview," *Microbes and Infection*, vol. 10, no. 9, pp. 1041–1050, 2008.

[22] A. Toledo-Arana, O. Dussurget, G. Nikitas, et al., "The *Listeria* transcriptional landscape from saprophytism to virulence," *Nature*, vol. 459, no. 7249, pp. 950–956, 2009.

[23] S. Mostowy and P. Cossart, "Cytoskeleton rearrangements during *Listeria* infection: clathrin and septins as new players in the game," *Cell Motility and the Cytoskeleton*, vol. 66, no. 10, pp. 816–823, 2009.

[24] R. S. Flannagan, G. Cosio, and S. Grinstein, "Antimicrobial mechanisms of phagocytes and bacterial evasion strategies," *Nature Reviews Microbiology*, vol. 7, no. 5, pp. 355–366, 2009.

[25] E. G. Pamer, "Immune responses to *Listeria monocytogenes*," *Nature Reviews Immunology*, vol. 4, no. 10, pp. 812–823, 2004.

[26] A. Wallecha, K. D. Carroll, P. C. Maciag, S. Rivera, V. Shahabi, and Y. Paterson, "Multiple effector mechanisms induced by recombinant *Listeria monocytogenes* anticancer immunotherapeutics," *Advances in Applied Microbiology*, vol. 66, pp. 1–27, 2009.

[27] Q. Jia, B.-Y. Lee, D. L. Clemens, R. A. Bowen, and M. A. Horwitz, "Recombinant attenuated *Listeria monocytogenes* vaccine expressing *Francisella tularensis* IglC induces protection in mice against aerosolized Type A *F. tularensis*," *Vaccine*, vol. 27, no. 8, pp. 1216–1229, 2009.

[28] D. G. Brockstedt and T. W. Dubensky Jr., "Promises and challenges for the development of *Listeria monocytogenes*-based immunotherapies," *Expert Review of Vaccines*, vol. 7, no. 7, pp. 1069–1084, 2008.

[29] Z. Li, M. Zhang, C. Zhou, X. Zhao, N. Iijima, and F. R. Frankel, "Novel vaccination protocol with two live mucosal vectors elicits strong cell-mediated immunity in the vagina and protects against vaginal virus challenge," *Journal of Immunology*, vol. 180, no. 4, pp. 2504–2513, 2008.

[30] M. T. Orr, N. N. Orgun, C. B. Wilson, and S. S. Way, "Cutting edge: recombinant *Listeria monocytogenes* expressing a single immune-dominant peptide confers protective immunity to herpes simplex virus-1 infection," *Journal of Immunology*, vol. 178, no. 8, pp. 4731–4735, 2007.

[31] D. Schlüter, E. Domann, C. Buck, et al., "Phosphatidylcholine-specific phospholipase C from *Listeria monocytogenes* is an important virulence factor in murine cerebral listeriosis," *Infection and Immunity*, vol. 66, no. 12, pp. 5930–5938, 1998.

[32] N. Autret, I. Dubail, P. Trieu-Cuot, P. Berche, and A. Charbit, "Identification of new genes involved in the virulence of *Listeria monocytogenes* by signature-tagged transposon mutagenesis," *Infection and Immunity*, vol. 69, no. 4, pp. 2054–2065, 2001.

[33] M. A. Pohl, M. Wiedmann, and K. K. Nightingale, "Associations among *Listeria monocytogenes* genotypes and distinct clinical manifestations of listeriosis in cattle," *American Journal of Veterinary Research*, vol. 67, no. 4, pp. 616–626, 2006.

[34] J. McLauchlin and D. Jones, "Erysipelothrix and Listeria," in *Topley and Wilson's Microbiology and Microbial Infections*, S. P. Borellio and B. I. Duerden, Eds., vol. 2, chapter 30, pp. 683–708, Arnold, London, UK, 9th edition, 1999.

[35] G. Domínguez-Bernal, S. Müller-Altrock, B. González-Zorn, et al., "A spontaneous genomic deletion in *Listeria ivanovii* identifies LIPI-2, a species-specific pathogenicity island encoding sphingomyelinase and numerous internalins," *Molecular Microbiology*, vol. 59, no. 2, pp. 415–432, 2006.

[36] M. W. Schmid, E. Y. W. Ng, R. Lampidis, et al., "Evolutionary history of the genus *Listeria* and its virulence genes," *Systematic and Applied Microbiology*, vol. 28, no. 1, pp. 1–18, 2005.

[37] J. A. Vazquez-Boland, G. Dominguez-Bernal, B. Gonzalez-Zorn, J. Kreft, and W. Goebel, "Pathogenicity islands and virulence evolution in *Listeria*," *Microbes and Infection*, vol. 3, no. 7, pp. 571–584, 2001.

[38] J. Rocourt, H. Hof, A. Schrettenbrunner, R. Malinverni, and J. Bille, "Acute purulent meningitis due to *Listeria seeligeri* in an immunocompetent adult," *Schweizerische Medizinische Wochenschrift*, vol. 116, no. 8, pp. 248–251, 1986.

[39] A. Rapose, S. D. Lick, and N. Ismail, "*Listeria grayi* bacteremia in a heart transplant recipient," *Transplant Infectious Disease*, vol. 10, no. 6, pp. 434–436, 2008.

[40] M. Perrin, M. Bemer, and C. Delamare, "Fatal case of *Listeria innocua* bacteremia," *Journal of Clinical Microbiology*, vol. 41, no. 11, pp. 5308–5309, 2003.

[41] K. Schwaiger, B. Stierstorfer, W. Schmahl, S. Lehmann, P. Gallien, and J. Bauer, "Survey on bacterial CNS infections in roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*) in Bavaria," *Berliner und Münchener Tierarztliche Wochenschrift*, vol. 118, no. 1-2, pp. 45–51, 2005.

[42] J. K. Walker, J. H. Morgan, J. McLauchlin, K. A. Grant, and J. A. Shallcross, "*Listeria innocua* isolated from a case of ovine meningoencephalitis," *Veterinary Microbiology*, vol. 42, no. 2-3, pp. 245–253, 1994.

[43] N. Mauder, R. Ecke, S. Mertins, et al., "Species-specific differences in the activity of PrfA, the key regulator of listerial virulence genes," *Journal of Bacteriology*, vol. 188, no. 22, pp. 7941–7956, 2006.

[44] R. D. Sleator, C. G. M. Gahan, and C. Hill, "A postgenomic appraisal of osmotolerance in *Listeria monocytogenes*," *Applied and Environmental Microbiology*, vol. 69, no. 1, pp. 1–9, 2003.

[45] F. Abram, E. Starr, K. A. G. Karatzas, et al., "Identification of components of the sigma B regulon in *Listeria monocytogenes* that contribute to acid and salt tolerance," *Applied and Environmental Microbiology*, vol. 74, no. 22, pp. 6848–6858, 2008.

[46] M. K. Borucki, J. D. Peppin, D. White, F. Loge, and D. R. Call, "Variation in biofilm formation among strains of *Listeria monocytogenes*," *Applied and Environmental Microbiology*, vol. 69, no. 12, pp. 7336–7342, 2003.

[47] J. Rocourt, A. Hogue, H. Toyofuku, C. Jacquet, and J. Schlundt, "*Listeria* and listeriosis: risk assessment as a new tool to unravel a multifaceted problem," *American Journal of Infection Control*, vol. 29, no. 4, pp. 225–227, 2001.

[48] A. J. Roberts and M. Wiedmann, "Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis," *Cellular and Molecular Life Sciences*, vol. 60, no. 5, pp. 904–918, 2003.

[49] J. McLauchlin, "*Listeria monocytogenes*, recent advances in the taxonomy and epidemiology of listeriosis in humans," *Journal of Applied Bacteriology*, vol. 63, no. 1, pp. 1–11, 1987.

[50] A. Oevermann, C. Botteron, T. Seuberlich, et al., "Neuropathological survey of fallen stock: active surveillance reveals high prevalence of encephalitic listeriosis in small ruminants," *Veterinary Microbiology*, vol. 130, no. 3-4, pp. 320–329, 2008.

[51] I. A. Gillespie, J. McLauchlin, K. A. Grant, et al., "Changing pattern of human listeriosis, England and Wales, 2001–2004," *Emerging Infectious Diseases*, vol. 12, no. 9, pp. 1361–1366, 2006.

[52] I. A. Gillespie, J. McLauchlin, C. L. Little, et al., "Disease presentation in relation to infection foci for non-pregnancy-associated human listeriosis in England and Wales, 2001 to 2007," *Journal of Clinical Microbiology*, vol. 47, no. 10, pp. 3301–3307, 2009.

[53] "The Community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007," *The EFSA Journal*, vol. 223, pp. 1–215, 2009.

[54] Centers for Disease Control and Prevention (CDC), "Outbreak of *Listeria monocytogenes* infections associated with pasteurized milk from a local dairy—Massachusetts, 2007," *Morbidity and Mortality Weekly Report*, vol. 57, no. 40, pp. 1097–1100, 2008.

[55] M. Vit, R. Olejnik, J. Dlhý, et al., "Outbreak of listeriosis in the Czech Republic, late 2006—preliminary report," *Euro Surveillance*, vol. 12, no. 2, Article ID E070208, 2007.

[56] S. Kathariou, L. Graves, C. Buchrieser, P. Glaser, R. M. Siletzky, and B. Swaminathan, "Involvement of closely related strains of a new clonal group of *Listeria monocytogenes* in the 1998-1999 and 2002 multistate outbreaks of foodborne listeriosis in the United States," *Foodborne Pathogens and Disease*, vol. 3, no. 3, pp. 292–302, 2006.

[57] P. S. Mead, L. Slutsker, V. Dietz, et al., "Food-related illness and death in the United States," *Emerging Infectious Diseases*, vol. 5, no. 5, pp. 607–625, 1999.

[58] J. Bille, D. S. Blanc, H. Schmid, et al., "Outbreak of human listeriosis associated with tomme cheese in northwest Switzerland, 2005," *Euro Surveillance*, vol. 11, no. 6, pp. 91–93, 2006.

[59] J. McLauchlin, "Human listeriosis in Britain, 1967–1985, a summary of 722 cases: 2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence," *Epidemiology and Infection*, vol. 104, no. 2, pp. 191–201, 1990.

[60] M. L. Paul, D. E. Dwyer, C. Chow, et al., "Listeriosis—a review of eighty-four cases," *Medical Journal of Australia*, vol. 160, no. 8, pp. 489–493, 1994.

[61] C. E. Cherubin, M. D. Appleman, P. N. R. Heseltine, W. Khayr, and C. W. Stratton, "Epidemiological spectrum and current treatment of listeriosis," *Reviews of Infectious Diseases*, vol. 13, no. 6, pp. 1108–1114, 1991.

[62] K. Skogberg, J. Syrjanen, M. Jahkola, et al., "Clinical presentation and outcome of listeriosis in patients with and without immunosuppressive therapy," *Clinical Infectious Diseases*, vol. 14, no. 4, pp. 815–821, 1992.

[63] E. M. Jones, S. Y. McCulloch, D. S. Reeves, and A. P. MacGowan, "A 10 year survey of the epidemiology and clinical aspects of listeriosis in a provincial English city," *Journal of Infection*, vol. 29, no. 1, pp. 91–103, 1994.

[64] J. Nolla-Salas, J. M. Anto, M. Almela, et al., "Incidence of listeriosis in Barcelona, Spain, in 1990. The Collaborative Study Group of Listeriosis of Barcelona," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 12, no. 3, pp. 157–161, 1993.

[65] B. G. Gellin and C. V. Broome, "Listeriosis," *Journal of the American Medical Association*, vol. 261, no. 9, pp. 1313–1320, 1989.

[66] T. L. Jackson, S. J. Eykyn, E. M. Graham, and M. R. Stanford, "Endogenous bacterial endophthalmitis: a 17-year prospective series and review of 267 reported cases," *Survey of Ophthalmology*, vol. 48, no. 4, pp. 403–423, 2003.

[67] K. Kida, N. Osada, K. Isahaya, et al., "*Listeria endocarditis* with acute thoracoabdominal aortic dissection," *Internal Medicine*, vol. 46, no. 15, pp. 1209–1212, 2007.

[68] K. Jayaraj, A. M. Di Bisceglie, and S. Gibson, "Spontaneous bacterial peritonitis caused by infection with *Listeria monocytogenes*: a case report and review of the literature," *American Journal of Gastroenterology*, vol. 93, no. 9, pp. 1556–1558, 1998.

[69] V. Vargas, C. Alemán, I. De Torres, et al., "*Listeria monocytogenes*-associated acute hepatitis in a liver transplant recipient," *Liver*, vol. 18, no. 3, pp. 213–215, 1998.

[70] M. Garcia-Montero, J. L. Rodriguez-Garcia, P. Calyo, et al., "Pneumonia caused by *Listeria monocytogenes*," *Respiration*, vol. 62, no. 2, pp. 107–109, 1995.

[71] L. Mereghetti, N. Marquet-van der Mee, P. Laudat, J. Loulergue, J. Jeannou, and A. Audurier, "*Listeria monocytogenes* septic arthritis in a natural joint: report of a case and review," *Clinical Microbiology and Infection*, vol. 4, no. 3, pp. 165–168, 1998.

[72] G. Revathi, A. Suneja, V. Talwar, and N. Aggarwal, "Fatal pericarditis due to *Listeria monocytogenes*," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 14, no. 3, pp. 254–255, 1995.

[73] M. L. Durand, S. B. Calderwood, D. J. Weber, et al., "Acute bacterial meningitis in adults—a review of 493 episodes," *New England Journal of Medicine*, vol. 328, no. 1, pp. 21–28, 1993.

[74] A. Schuchat, K. Robinson, J. D. Wenger, et al., "Bacterial meningitis in the United States in 1995. Active Surveillance Team," *New England Journal of Medicine*, vol. 337, no. 14, pp. 970–976, 1997.

[75] B. Sigurdardottir, O. M. Bjornsson, K. E. Jonsdottir, H. Erlendsdottir, and S. Gudmundsson, "Acute bacterial meningitis in adults: a 20-year overview," *Archives of Internal Medicine*, vol. 157, no. 4, pp. 425–430, 1997.

[76] B. G. Gellin, C. V. Broome, W. F. Bibb, R. E. Weaver, S. Gaventa, and L. Mascola, "The epidemiology of listeriosis in the United States—1986. Listeriosis Study Group," *American Journal of Epidemiology*, vol. 133, no. 4, pp. 392–401, 1991.

[77] V. Goulet and P. Marchetti, "Listeriosis in 225 non-pregnant patients in 1992: clinical aspects and outcome in relation to predisposing conditions," *Scandinavian Journal of Infectious Diseases*, vol. 28, no. 4, pp. 367–374, 1996.

[78] A. S. Hussein and S. D. Shafran, "Acute bacterial meningitis in adults: a 12-year review," *Medicine*, vol. 79, no. 6, pp. 360–368, 2000.

[79] M. H. Kyaw, P. Christie, I. G. Jones, and H. Campbell, "The changing epidemiology of bacterial meningitis and invasive non-meningitic bacterial disease in Scotland during

the period 1983–1999," *Scandinavian Journal of Infectious Diseases*, vol. 34, no. 4, pp. 289–298, 2002.

[80] E. Mylonakis, E. L. Hohmann, and S. B. Calderwood, "Central nervous system infection with *Listeria monocytogenes*: 33 years' experience at a general hospital and review of 776 episodes from the literature," *Medicine*, vol. 77, no. 5, pp. 313–336, 1998.

[81] E.-A. Antal, E. Dietrichs, E. M. Loberg, K. K. Melby, and J. Maehlen, "Brain stem encephalitis in listeriosis," *Scandinavian Journal of Infectious Diseases*, vol. 37, no. 3, pp. 190–194, 2005.

[82] R. W. Armstrong and P. C. Fung, "Brainstem encephalitis (rhombencephalitis) due to *Listeria monocytogenes*: case report and review," *Clinical Infectious Diseases*, vol. 16, no. 5, pp. 689–702, 1993.

[83] S. S. Pollock, T. M. Pollock, and M. J. G. Harrison, "Infection of the central nervous system by *Listeria monocytogenes*: a review of 54 adult and juvenile cases," *Quarterly Journal of Medicine*, vol. 53, no. 211, pp. 331–340, 1984.

[84] H. Eck, "Encephalomyelitis listeriaca apostematosa," *Schweizerische Medizinische Wochenschrift*, vol. 87, no. 9, pp. 210–214, 1957.

[85] P.-A. Uldry, T. Kuntzer, J. Bogousslavsky, et al., "Early symptoms and outcome of *Listeria monocytogenes* rhombencephalitis: 14 adult cases," *Journal of Neurology*, vol. 240, no. 4, pp. 235–242, 1993.

[86] F. Gray and J. M. Alonso, "Bacterial infections of the central nervous system," in *Greenfield's Neuropathology*, D. I. Graham and P. L. Lantos, Eds., pp. 151–193, Arnold, London, UK, 7th edition, 2002.

[87] E.-A. Antal, E.-M. Loberg, E. Dietrichs, and J. Maehlen, "Neuropathological findings in 9 cases of *Listeria monocytogenes* brain stem encephalitis," *Brain Pathology*, vol. 15, no. 3, pp. 187–191, 2005.

[88] J. A. Crump, P. M. Griffin, and F. J. Angulo, "Bacterial contamination of animal feed and its relationship to human foodborne illness," *Clinical Infectious Diseases*, vol. 35, no. 7, pp. 859–865, 2002.

[89] F. Driehuis and S. J. W. H. Oude Elferink, "The impact of the quality of silage on animal health and food safety: a review," *Veterinary Quarterly*, vol. 22, no. 4, pp. 212–216, 2000.

[90] H. Ueno, K. Yokota, T. Arai, et al., "The prevalence of *Listeria monocytogenes* in the environment of dairy farms," *Microbiology and Immunology*, vol. 40, no. 2, pp. 121–124, 1996.

[91] T. Yoshida, Y. Kato, M. Sato, and K. Hirai, "Sources and routes of contamination of raw milk with *Listeria monocytogenes* and its control," *Journal of Veterinary Medical Science*, vol. 60, no. 10, pp. 1165–1168, 1998.

[92] H. O. Mohammed, K. Stipetic, P. L. McDonough, R. N. Gonzalez, D. V. Nydam, and E. R. Atwill, "Identification of potential on-farm sources of *Listeria monocytogenes* in herds of dairy cattle," *American Journal of Veterinary Research*, vol. 70, no. 3, pp. 383–388, 2009.

[93] M. Sanaa, B. Poutrel, J. L. Menard, and F. Serieys, "Risk factors associated with contamination of raw milk by *Listeria monocytogenes* in dairy farms," *Journal of Dairy Science*, vol. 76, no. 10, pp. 2891–2898, 1993.

[94] M. L. Gray, "Isolation of *Listeria monocytogenes* from oat silage," *Science*, vol. 132, no. 3441, pp. 1767–1768, 1960.

[95] M. L. Gray, "A possible link in the relationship between silage feeding and listeriosis," *Journal of the American Veterinary Medical Association*, vol. 136, no. 5, pp. 205–208, 1960.

[96] D. R. Fenlon, "Wild birds and silage as reservoirs of *Listeria* in the agricultural environment," *Journal of Applied Bacteriology*, vol. 59, no. 6, pp. 537–543, 1985.

[97] A. S. Donald, D. R. Fenlon, and B. Seddon, "The relationship between ecophysiology, indigenous microflora and growth of *Listeria monocytogenes* in grass silage," *Journal of Applied Bacteriology*, vol. 79, no. 2, pp. 141–148, 1995.

[98] J. A. Vázquez-Boland, L. Dominguez, M. Blanco, et al., "Epidemiologic investigation of a silage-associated epizootic of ovine listeric encephalitis, using a new *Listeria*-selective enumeration medium and phage typing," *American Journal of Veterinary Research*, vol. 53, no. 3, pp. 368–371, 1992.

[99] E. T. Ryser, S. M. Arimi, and C. W. Donnelly, "Effects of pH on distribution of *Listeria* ribotypes in corn, hay, and grass silage," *Applied and Environmental Microbiology*, vol. 63, no. 9, pp. 3695–3697, 1997.

[100] A. I. Vela, J. F. Fernandez-Garayzabal, J. A. Vazquez, et al., "Molecular typing by pulsed-field gel electrophoresis of Spanish animal and human *Listeria monocytogenes* isolates," *Applied and Environmental Microbiology*, vol. 67, no. 12, pp. 5840–5843, 2001.

[101] M. Wiedmann, J. Czajka, N. Bsat, et al., "Diagnosis and epidemiological association of *Listeria monocytogenes* strains in two outbreaks of listerial encephalitis in small ruminants," *Journal of Clinical Microbiology*, vol. 32, no. 4, pp. 991–996, 1994.

[102] J. W. Wilesmith and M. Gitter, "Epidemiology of ovine listeriosis in Great Britain," *Veterinary Record*, vol. 119, no. 19, pp. 467–470, 1986.

[103] H. Gronstol, "Listeriosis in sheep. Isolation of *Listeria monocytogenes* from grass silage," *Acta Veterinaria Scandinavica*, vol. 20, no. 4, pp. 492–497, 1979.

[104] M. Wiedmann, T. Arvik, J. L. Bruce, et al., "Investigation of a listeriosis epizootic in sheep in New York state," *American Journal of Veterinary Research*, vol. 58, no. 7, pp. 733–737, 1997.

[105] J. C. Low and C. P. Renton, "Septicaemia, encephalitis and abortions in a housed flock of sheep caused by *Listeria monocytogenes* type 1/2," *Veterinary Record*, vol. 116, no. 6, pp. 147–150, 1985.

[106] D. R. Fenlon, J. Wilson, and W. Donachie, "The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing," *Journal of Applied Bacteriology*, vol. 81, no. 6, pp. 641–650, 1996.

[107] A. J. Ho, R. Ivanek, Y. T. Grohn, K. K. Nightingale, and M. Wiedmann, "*Listeria monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation and includes outbreaks and sporadic cases of shedding of specific *L. monocytogenes* subtypes," *Preventive Veterinary Medicine*, vol. 80, no. 4, pp. 287–305, 2007.

[108] R. Fensterbank, A. Audurier, J. Godu, P. Guerrault, and N. Malo, "*Listeria* strains isolated from sick animals and consumed silage," *Annales de Recherches Veterinaires*, vol. 15, no. 1, pp. 113–118, 1984.

[109] F. Baxter, F. Wright, R. M. Chalmers, J. C. Low, and W. Donachie, "Characterization by multilocus enzyme electrophoresis of *Listeria monocytogenes* isolates involved in ovine listeriosis outbreaks in Scotland from 1989 to 1991," *Applied and Environmental Microbiology*, vol. 59, no. 9, pp. 3126–3129, 1993.

[110] M. Wiedmann, S. Mobini, J. R. Cole Jr., C. K. Watson, G. T. Jeffers, and K. J. Boor, "Molecular investigation of a listeriosis outbreak in goats caused by an unusual strain of *Listeria*

*monocytogenes*," *Journal of the American Veterinary Medical Association*, vol. 215, no. 3, pp. 369–371, 1999.

[111] R. Vandegraaff, N. A. Borland, and J. W. Browning, "An outbreak of listerial meningo-encephalitis in sheep," *Australian Veterinary Journal*, vol. 57, no. 2, pp. 94–96, 1981.

[112] L. Belchev, "*Listeria* enzootic in newborn lambs," *Veterinarno-Meditsinski Nauki*, vol. 16, no. 3, pp. 57–62, 1979.

[113] H. Kumar, B. B. Singh, M. S. Bal, et al., "Pathological and epidemiological investigations into listerial encephalitis in sheep," *Small Ruminant Research*, vol. 71, no. 1–3, pp. 293–297, 2007.

[114] D. Heim, R. Fatzer, B. Hornlimann, and M. Vandevelde, "Frequency of neurological diseases in cattle," *Schweizer Archiv für Tierheilkunde*, vol. 139, no. 8, pp. 354–362, 1997.

[115] M. Jeffrey, "A neuropathological survey of brains submitted under the Bovine Spongiform Encephalopathy Orders in Scotland," *Veterinary Record*, vol. 131, no. 15, pp. 332–337, 1992.

[116] I. S. McGill and G. A. H. Wells, "Neuropathological findings in cattle with clinically suspect but histologically unconfirmed bovine spongiform encephalopathy (BSE)," *Journal of Comparative Pathology*, vol. 108, no. 3, pp. 241–260, 1993.

[117] M. Miyashita, B. Stierstorfer, and W. Schmahl, "Neuropathological findings in brains of Bavarian cattle clinically suspected of bovine spongiform encephalopathy," *Journal of Veterinary Medicine Series B*, vol. 51, no. 5, pp. 209–215, 2004.

[118] P. Boerlin and J.-C. Piffaretti, "Typing of human, animal, food, and environmental isolates of *Listeria monocytogenes* by multilocus enzyme electrophoresis," *Applied and Environmental Microbiology*, vol. 57, no. 6, pp. 1624–1629, 1991.

[119] M. K. Borucki, J. Reynolds, C. C. Gay, et al., "Dairy farm reservoir of *Listeria monocytogenes* sporadic and epidemic strains," *Journal of Food Protection*, vol. 67, no. 11, pp. 2496–2499, 2004.

[120] K. K. Nightingale, Y. H. Schukken, C. R. Nightingale, et al., "Ecology and transmission of *Listena monocytogenes* infecting ruminants and in the farm environment," *Applied and Environmental Microbiology*, vol. 70, no. 8, pp. 4458–4467, 2004.

[121] O. Okwumabua, M. O'Connor, E. Shull, et al., "Characterization of *Listeria monocytogenes* isolates from food animal clinical cases: PFGE pattern similarity to strains from human listeriosis cases," *FEMS Microbiology Letters*, vol. 249, no. 2, pp. 275–281, 2005.

[122] M. Gitter, "Listeriosis in farm animals in Great Britain," in *Isolation and Identification of Microorganisms of Medical and Veterinary Importance*, C. H. Collins and J. M. Grange, Eds., pp. 191–200, Academic Press, New York, NY, USA, 1985.

[123] J. W. McDonald, "An outbreak of abortion due to *Listeria monocytogenes* in an experimental flock of sheep," *Australian Veterinary Journal*, vol. 43, no. 12, pp. 564–567, 1967.

[124] P. W. Ladds, S. M. Dennis, and R. F. Cooper, "Sequential studies of experimentally induced ovine listerial abortion: clinical changes and bacteriologic examinations," *American Journal of Veterinary Research*, vol. 35, no. 2, pp. 155–160, 1974.

[125] D. D. Wardrope and N. S. MacLeod, "Outbreak of *Listeria* meningoencephalitis in young lambs," *Veterinary Record*, vol. 113, no. 10, pp. 213–214, 1983.

[126] J. C. Low, F. Wright, J. McLauchlin, and W. Donachie, "Serotyping and distribution of *Listeria* isolates from cases of ovine listeriosis," *Veterinary Record*, vol. 133, no. 7, pp. 165–166, 1993.

[127] M. Wagner, D. Melzner, Z. Bagò, et al., "Outbreak of clinical listeriosis in sheep: evaluation from possible contamination routes from feed to raw produce and humans," *Journal of Veterinary Medicine Series B*, vol. 52, no. 6, pp. 278–283, 2005.

[128] J. R. Husu, "Epidemiological studies on the occurrence of *Listeria monocytogenes* in the feces of dairy cattle," *Zentralblatt für Veterinarmedizin B*, vol. 37, no. 4, pp. 276–282, 1990.

[129] A. N. Pell, "Manure and microbes: public and animal health problem?" *Journal of Dairy Science*, vol. 80, no. 10, pp. 2673–2681, 1997.

[130] N. Skovgaard and C.-A. Morgen, "Detection of *Listeria* spp. in faeces from animals, in feeds, and in raw foods of animal origin," *International Journal of Food Microbiology*, vol. 6, no. 3, pp. 229–242, 1988.

[131] K. M. Charlton and M. M. Garcia, "Spontaneous listeric encephalitis and neuritis in sheep. Light microscopic studies," *Veterinary Pathology*, vol. 14, no. 4, pp. 297–313, 1977.

[132] D. A. Gill, ""Circling" disease of sheep in New Zealand," *Veterinary Journal*, vol. 87, pp. 60–74, 1931.

[133] D. A. Gill, ""Circling" disease: a meningoencephalitis of sheep in New Zealand," *Veterinary Journal*, vol. 89, pp. 258–270, 1933.

[134] G. A. Gates, D. C. Blenden, and L. D. Kintner, "Listeric myelitis in sheep," *Journal of the American Veterinary Medical Association*, vol. 150, no. 2, pp. 200–204, 1967.

[135] R. M. Barlow and B. McGorum, "Ovine listerial encephalitis: analysis, hypothesis and synthesis," *Veterinary Record*, vol. 116, no. 9, pp. 233–236, 1985.

[136] A. Otter and W. F. Blakemore, "Observation on the presence of *Listeria monocytogenes* in axons," *Acta Microbiologica Hungarica*, vol. 36, no. 2-3, pp. 125–131, 1989.

[137] M. Peters, J. Pohlenz, K. Jaton, B. Ninet, and J. Bille, "Studies of the detection of *Listeria monocytogenes* by culture and PCR in cerebrospinal fluid samples from ruminants with listeric encephalitis," *Zentralblatt für Veterinarmedizin B*, vol. 42, no. 2, pp. 84–88, 1995.

[138] G. C. Johnson, C. W. Maddox, W. H. Fales, et al., "Epidemiologic evaluation of encephalitic listeriosis in goats," *Journal of the American Veterinary Medical Association*, vol. 208, no. 10, pp. 1695–1699, 1996.

[139] H. M. Erdogan, B. Cetinkaya, L. E. Green, P. J. Cripps, and K. L. Morgan, "Prevalence, incidence, signs and treatment of clinical listeriosis in dairy cattle in England," *Veterinary Record*, vol. 149, no. 10, pp. 289–293, 2001.

[140] P. Boerlin, F. Boerlin-Petzold, and T. Jemmi, "Use of listeriolysin O and internalin A in a seroepidemiological study of listeriosis in Swiss dairy cows," *Journal of Clinical Microbiology*, vol. 41, no. 3, pp. 1055–1061, 2003.

[141] M. Gitter, S. Terlecki, and P. A. Turnbull, "An outbreak of visceral and cerebral listeriosis in a flock of sheep in south east England," *The Veterinary Record*, vol. 77, no. 1, pp. 11–15, 1965.

[142] G. Pallaske, "Über das Vorkommen einer seuchenhaften Encephalomyelitis purulenta bei Schafen in Deutschland (Listerella-Infektion)," *Berliner und Münchener Tierarztliche Wochenschrift*, vol. 37, pp. 441–445, 1940.

[143] W. Krüger, "Das Vorkommen von *Listeria monocytogenes* in den verschiedenen Silagen und dessen ätiologische Bedeutung," *Archiv für Experimentelle Veterinärmedizin*, vol. 17, no. 1, pp. 181–203, 1963.

[144] D. A. Gill, "Ovine bacterial encephalitis (Circling disease) and the genus *Listerella*," *Australian Veterinary Journal*, vol. 13, no. 2, pp. 46–56, 1937.

[145] H. E. Biester and L. H. Schwartz, "Studies on *Listerella* infection in sheep," *The Journal of Infectious Diseases*, vol. 64, pp. 135–144, 1939.

[146] P. Olafson, "*Listerella* encephalitis (circling disease) of sheep, cattle and goats," *Cornell Veterinarian*, vol. 30, no. 2, pp. 141–150, 1940.

[147] R. Graham, G. L. Dunlap, and C. A. Brandly, "Ovine and bovine listerellosis in Illinois," *Science*, vol. 88, no. 2277, pp. 171–172, 1938.

[148] J. Brugère-Picoux, "Ovine listeriosis," *Small Ruminant Research*, vol. 76, no. 1-2, pp. 12–20, 2008.

[149] H. Gronstol, "Listeriosis in sheep. Experimental listeric infection in sheep treated with various immunosuppressiva," *Acta Veterinaria Scandinavica*, vol. 21, no. 3, pp. 415–427, 1980.

[150] H. Gronstol and J. Overas, "Listeriosis in sheep. *Eperythrozoon ovis* infection used as a model to study predisposing factors," *Acta Veterinaria Scandinavica*, vol. 21, no. 4, pp. 523–532, 1980.

[151] H. Gronstol and J. Overas, "Listeriosis in sheep. Tick-borne fever used as a model to study predisposing factors," *Acta Veterinaria Scandinavica*, vol. 21, no. 4, pp. 533–545, 1980.

[152] W. Jakob, "Further experimental studies on the pathogenesis of cerebral listeriosis of the sheep. II. The course of the experimental infection with *Listeria monocytogenes* under various stress conditions," *Archiv für Experimentelle Veterinarmedizin*, vol. 21, no. 3, pp. 675–684, 1967.

[153] W. Jakob, "Untersuchungen zum Infektionsmodus und zur Pathogenese der Listeriose des Schafes," *Archiv für Experimentelle Veterinärmedizin*, vol. 17, no. 5, pp. 1081–1128, 1963.

[154] C. Olson and D. Segre, "An agent enhancing listeriosis of sheep," *American Journal of Veterinary Research*, vol. 17, no. 63, pp. 235–242, 1956.

[155] C. C. Olson, O. D. Grace, D. Segre, and I. C. Blore, "Enhancement of listeriosis in sheep with material from bovine mucosal disease," *American Journal of Veterinary Research*, vol. 18, pp. 303–309, 1957.

[156] R. Malinverni, M. P. Glauser, J. Bille, and J. Rocourt, "Unusual clinical features of an epidemic of listeriosis associated with a particular phage type," *European Journal of Clinical Microbiology*, vol. 5, no. 2, pp. 169–171, 1986.

[157] W. Jakob, "Further experimental studies on the pathogenesis of cerebral listeriosis in sheep. 3. The course of the experimental infection with L. monocytogenes in sheep with high protein and low protein nutrition," *Archiv für Experimentelle Veterinarmedizin*, vol. 21, no. 4, pp. 1061–1072, 1967.

[158] A. Wuilleret, P. Despres, L. Monteiro, C. Bouzakoura, and E. Wildi, "Bacteriological and histological diagnosis of listeriosis during an ovine enzootic," *Schweizer Archiv für Tierheilkunde*, vol. 111, no. 11, pp. 622–641, 1969.

[159] Y. Akiyama, O. Asahi, and T. Hosoda, "Studies on the mechanism of infection of the brain with *Listeria monocytogenes*," *American Journal of Veterinary Research*, vol. 18, no. 66, pp. 147–157, 1957.

[160] U. Braun, C. Stehle, and F. Ehrensperger, "Clinical findings and treatment of listeriosis in 67 sheep and goats," *Veterinary Record*, vol. 150, no. 2, pp. 38–42, 2002.

[161] G. Schweizer, F. Ehrensperger, P. R. Torgerson, and U. Braun, "Clinical findings and treatment of 94 cattle presumptively diagnosed with listeriosis," *Veterinary Record*, vol. 158, no. 17, pp. 588–592, 2006.

[162] J. C. Low, F. Wright, J. McLauchlin, and W. Donachie, "Serotyping and distribution of Listeria isolates from cases of ovine listeriosis," *Veterinary Record*, vol. 133, no. 7, pp. 165–166, 1993.

[163] J. T. Seaman, G. I. Carter, M. J. Carrigan, and F. A. Cockram, "An outbreak of listerial myelitis in sheep," *Australian Veterinary Journal*, vol. 67, no. 4, pp. 142–143, 1990.

[164] G. Schweizer, B. Fuhrer, and U. Braun, "Signs of spinal cord disease in two heifers caused by *Listeria monocytogenes*," *Veterinary Record*, vol. 154, no. 2, pp. 54–55, 2004.

[165] A. Oevermann, P. S. Di, M. G. Doherr, et al., "Neuropathogenesis of naturally occurring encephalitis caused by *Listeria monocytogenes* in ruminants," *Brain Pathology*, vol. 20, no. 2, pp. 378–390, 2010.

[166] D. R. Cordy and J. W. Osebold, "The neuropathogenesis of listeria encephalomyelitis in sheep and mice," *The Journal of Infectious Diseases*, vol. 104, no. 2, pp. 164–173, 1959.

[167] A. Camejo, C. Buchrieser, E. Couvé, et al., "In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection," *PLoS Pathogens*, vol. 5, no. 5, Article ID e1000449, 2009.

[168] P. Cossart and P. J. Sansonetti, "Bacterial invasion: the paradigms of enteroinvasive pathogens," *Science*, vol. 304, no. 5668, pp. 242–248, 2004.

[169] P. Glaser, L. Frangeul, C. Buchrieser, et al., "Comparative genomics of *Listeria* species," *Science*, vol. 294, no. 5543, pp. 849–852, 2001.

[170] K. E. Nelson, D. E. Fouts, E. F. Mongodin, et al., "Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species," *Nucleic Acids Research*, vol. 32, no. 8, pp. 2386–2395, 2004.

[171] H. Bierne, C. Sabet, N. Personnic, and P. Cossart, "Internalins: a complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*," *Microbes and Infection*, vol. 9, no. 10, pp. 1156–1166, 2007.

[172] D. Cabanes, P. Dehoux, O. Dussurget, L. Frangeul, and P. Cossart, "Surface proteins and the pathogenic potential of *Listeria monocytogenes*," *Trends in Microbiology*, vol. 10, no. 5, pp. 238–245, 2002.

[173] J.-L. Gaillard, P. Berche, C. Frehel, E. Gouin, and P. Cossart, "Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci," *Cell*, vol. 65, no. 7, pp. 1127–1141, 1991.

[174] S. Dramsi, I. Biswas, E. Maguin, L. Braun, P. Mastroeni, and P. Cossart, "Entry of *Listeria monocytogenes* into hepatocytes requires expression of inIB, a surface protein of the internalin multigene family," *Molecular Microbiology*, vol. 16, no. 2, pp. 251–261, 1995.

[175] M. Lecuit, S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart, "A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*," *EMBO Journal*, vol. 18, no. 14, pp. 3956–3963, 1999.

[176] M. Lecuit, S. Vandormael-Pournin, J. Lefort, et al., "A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier," *Science*, vol. 292, no. 5522, pp. 1722–1725, 2001.

[177] M. Lecuit, D. M. Nelson, S. D. Smith, et al., "Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: role of internalin interaction with trophoblast E-cadherin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 16, pp. 6152–6157, 2004.

[178] M. Lecuit, "Understanding how *Listeria monocytogenes* targets and crosses host barriers," *Clinical Microbiology and Infection*, vol. 11, no. 6, pp. 430–436, 2005.

[179] L. L. Rubin, D. E. Hall, S. Porter, et al., "A cell culture model of the blood-brain barrier," *Journal of Cell Biology*, vol. 115, no. 6, pp. 1725–1735, 1991.

[180] H. Bierne and P. Cossart, "InIB, a surface protein of *Listeria monocytogenes* that behaves as an invasin and a growth factor," *Journal of Cell Science*, vol. 115, no. 17, pp. 3357–3367, 2002.

[181] R. Jonquieres, J. Pizarro-Cerda, and P. Cossart, "Synergy between the N- and C-terminal domains of InlB for efficient invasion of non-phagocytic cells by *Listeria monocytogenes*," *Molecular Microbiology*, vol. 42, no. 4, pp. 955–965, 2001.

[182] L. Braun, B. Ghebrehiwet, and P. Cossart, "GC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*," *EMBO Journal*, vol. 19, no. 7, pp. 1458–1466, 2000.

[183] Y. Shen, M. Naujokas, M. Park, and K. Ireton, "InIB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase," *Cell*, vol. 103, no. 3, pp. 501–510, 2000.

[184] D. Liu, A. J. Ainsworth, F. W. Austin, and M. L. Lawrence, "Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes," *Journal of Medical Microbiology*, vol. 52, no. 12, pp. 1065–1070, 2003.

[185] D. Liu, M. L. Lawrence, F. W. Austin, and A. J. Ainsworth, "A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*," *Journal of Microbiological Methods*, vol. 71, no. 2, pp. 133–140, 2007.

[186] C. Sabet, A. Toledo-Arana, N. Personnic, et al., "The *Listeria monocytogenes* virulence factor InlJ is specifically expressed in vivo and behaves as an adhesin," *Infection and Immunity*, vol. 76, no. 4, pp. 1368–1378, 2008.

[187] C. Sabet, M. Lecuit, D. Cabanes, P. Cossart, and H. Bierne, "LPXTG protein InlJ, a newly identified internalin involved in *Listeria monocytogenes* virulence," *Infection and Immunity*, vol. 73, no. 10, pp. 6912–6922, 2005.

[188] F. Engelbrecht, S. K. Chun, C. Ochs, et al., "A new PrfA-regulated gene of *Listeria monocytogenes* encoding at small, secreted protein which belongs to the family of internalins," *Molecular Microbiology*, vol. 21, no. 4, pp. 823–837, 1996.

[189] B. Bergmann, D. Raffelsbauer, M. Kuhn, et al., "InlA- but not InlB-mediated internalization of *Listeria monocytogenes* by non-phagocytic mammalian cells needs the support of other internalins," *Molecular Microbiology*, vol. 43, no. 3, pp. 557–570, 2002.

[190] D. Raffelsbauer, A. Bubert, F. Engelbrecht, et al., "The gene cluster inlC2DE of *Listeria monocytogenes* contains additional new internalin genes and is important for virulence in mice," *Molecular and General Genetics*, vol. 260, no. 2-3, pp. 144–158, 1998.

[191] E. Veiga and P. Cossart, "*Listeria* hijacks the clathrin-dependent endocytic machinery to invade mammalian cells," *Nature Cell Biology*, vol. 7, no. 9, pp. 894–900, 2005.

[192] E. Veiga, J. A. Guttman, M. Bonazzi, et al., "Invasive and adherent bacterial pathogens co-Opt host clathrin for infection," *Cell Host and Microbe*, vol. 2, no. 5, pp. 340–351, 2007.

[193] C. Archambaud, M. A. Nahori, J. Pizarro-Cerda, P. Cossart, and O. Dussurget, "Control of *Listeria* superoxide dismutase by phosphorylation," *Journal of Biological Chemistry*, vol. 281, no. 42, pp. 31812–31822, 2006.

[194] I. G. Boneca, O. Dussurget, D. Cabanes, et al., "A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 3, pp. 997–1002, 2007.

[195] D. A. Portnoy, T. Chakraborty, W. Goebel, and P. Cossart, "Molecular determinants of *Listeria monocytogenes* pathogenesis," *Infection and Immunity*, vol. 60, no. 4, pp. 1263–1267, 1992.

[196] P. Schnupf and D. A. Portnoy, "Listeriolysin O: a phagosome-specific lysin," *Microbes and Infection*, vol. 9, no. 10, pp. 1176–1187, 2007.

[197] I. Chico-Calero, M. Suárez, B. González-Zorn, et al., "Hpt, a bacterial homolog of the microsomal glucose-6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 1, pp. 431–436, 2002.

[198] M. O'Riordan, M. A. Moors, and D. A. Portnoy, "*Listeria* intracellular growth and virulence require host-derived lipoic acid," *Science*, vol. 302, no. 5644, pp. 462–464, 2003.

[199] J. A. Vázquez-Boland, M. Kuhn, P. Berche, et al., "*Listeria* pathogenesis and molecular virulence determinants," *Clinical Microbiology Reviews*, vol. 14, no. 3, pp. 584–640, 2001.

[200] O. Dussurget, J. Pizarro-Cerda, and P. Cossart, "Molecular determinants of *Listeria monocytogenes* virulence," *Annual Review of Microbiology*, vol. 58, pp. 587–610, 2004.

[201] J. McLauchlin and J. C. Low, "Primary cutaneous listeriosis in adults: an occupational disease of veterinarians and farmers," *Veterinary Record*, vol. 135, no. 26, pp. 615–617, 1994.

[202] E. J. Regan, G. A. J. Harrison, S. Butler, et al., "Primary cutaneous listeriosis in a veterinarian," *Veterinary Record*, vol. 157, no. 7, p. 207, 2005.

[203] P. D. Cotter, C. G. M. Gahan, and C. Hill, "Analysis of the role of the *Listeria monocytogenes* F0F1-ATPase operon in the acid tolerance response," *International Journal of Food Microbiology*, vol. 60, no. 2-3, pp. 137–146, 2000.

[204] P. D. Cotter, C. G. M. Gahan, and C. Hill, "A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid," *Molecular Microbiology*, vol. 40, no. 2, pp. 465–475, 2001.

[205] M. Begley, R. D. Sleator, C. G. M. Gahan, and C. Hill, "Contribution of three bile-associated loci, bsh, pva, and btlB, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*," *Infection and Immunity*, vol. 73, no. 2, pp. 894–904, 2005.

[206] O. Dussurget, D. Cabanes, P. Dehoux, et al., "*Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis," *Molecular Microbiology*, vol. 45, no. 4, pp. 1095–1106, 2002.

[207] R. D. Sleator, H. H. Wemekamp-Kamphuis, C. G. M. Gahan, T. Abee, and C. Hill, "A PrfA-regulated bile exclusion system (BilE) is a novel virulence factor in *Listeria monocytogenes*," *Molecular Microbiology*, vol. 55, no. 4, pp. 1183–1195, 2005.

[208] J. Hardy, K. P. Francis, M. DeBoer, et al., "Extracellular replication of *Listeria monocytogenes* in the murine gall bladder," *Science*, vol. 303, no. 5659, pp. 851–853, 2004.

[209] O. Dussurget, "New insights into determinants of *Listeria monocytogenes* virulence," *International Review of Cell and Molecular Biology*, vol. 270, pp. 1–38, 2008.

[210] G. B. Mackaness, "Cellular resistance to infection," *The Journal of Experimental Medicine*, vol. 116, pp. 381–406, 1962.

[211] M. E. Munk and H. E. Kaufmann, "*Listeria monocytogenes* reactive T lymphocytes in healthy individuals," *Microbial Pathogenesis*, vol. 5, no. 1, pp. 49–54, 1988.

[212] O. Disson, S. Grayo, E. Huillet, et al., "Conjugated action of two species-specific invasion proteins for fetoplacental listeriosis," *Nature*, vol. 455, no. 7216, pp. 1114–1118, 2008.

[213] C. Jacquet, M. Doumith, J. I. Gordon, et al., "A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*," *Journal of Infectious Diseases*, vol. 189, no. 11, pp. 2094–2100, 2004.

[214] C. Buchrieser, C. Rusniok, F. Kunst, P. Cossart, and P. Glaser, "Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria innocua*: clues for evolution and pathogenicity," *FEMS Immunology and Medical Microbiology*, vol. 35, no. 3, pp. 207–213, 2003.

[215] C. Buchrieser, "Biodiversity of the species *Listeria monocytogenes* and the genus *Listeria*," *Microbes and Infection*, vol. 9, no. 10, pp. 1147–1155, 2007.

[216] T. Hain, C. Steinweg, C. T. Kuenne, et al., "Whole-genome sequence of *Listeria welshimeri* reveals common steps in genome reduction with *Listeria innocua* as compared to *Listeria monocytogenes*," *Journal of Bacteriology*, vol. 188, no. 21, pp. 7405–7415, 2006.

[217] E. Gouin, J. Mengaud, and P. Cossart, "The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a nonpathogenic species," *Infection and Immunity*, vol. 62, no. 8, pp. 3550–3553, 1994.

[218] M. Leimeister-Wachter, E. Domann, and T. Chakraborty, "The expression of virulence genes in *Listeria monocytogenes* is thermoregulated," *Journal of Bacteriology*, vol. 174, no. 3, pp. 947–952, 1992.

[219] J. Johansson, P. Mandin, A. Renzoni, et al., "An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*," *Cell*, vol. 110, no. 5, pp. 551–561, 2002.

[220] A. Renzoni, A. Klarsfeld, S. Dramsi, and P. Cossart, "Evidence that PrfA, the pleiotropic activator of virulence genes in *Listeria monocytogenes*, can be present but inactive," *Infection and Immunity*, vol. 65, no. 4, pp. 1515–1518, 1997.

[221] D. A. Drevets, P. J. M. Leenen, and R. A. Greenfield, "Invasion of the central nervous system by intracellular bacteria," *Clinical Microbiology Reviews*, vol. 17, no. 2, pp. 323–347, 2004.

[222] P. Berche, "Bacteremia is required for invasion of the murine central nervous system by *Listeria monocytogenes*," *Microbial Pathogenesis*, vol. 18, no. 5, pp. 323–336, 1995.

[223] S. López, A. J. Marco, N. Prats, and C. J. Czuprynski, "Critical role of neutrophils in eliminating *Listeria monocytogenes* from the central nervous system during experimental murine listeriosis," *Infection and Immunity*, vol. 68, no. 8, pp. 4789–4791, 2000.

[224] S. López, N. Prats, and A. J. Marco, "Expression of E-selectin, P-selectin, and intercellular adhesion molecule-1 during experimental murine listeriosis," *American Journal of Pathology*, vol. 155, no. 4, pp. 1391–1397, 1999.

[225] N. Prats, V. Briones, M. M. Blanco, et al., "Choroiditis and meningitis in experimental murine infection with *Listeria monocytogenes*," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 11, no. 8, pp. 744–747, 1992.

[226] U. Sibelius, T. Chakraborty, B. Krögel, et al., "The listerial exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C synergize to elicit endothelial cell phosphoinositide metabolism," *Journal of Immunology*, vol. 157, no. 9, pp. 4055–4060, 1996.

[227] U. Sibelius, F. Rose, T. Chakraborty, et al., "Listeriolysin is a potent inducer of the phosphatidylinositol response and lipid mediator generation in human endothelial cells," *Infection and Immunity*, vol. 64, no. 2, pp. 674–676, 1996.

[228] D. A. Drevets, R. T. Sawyer, T. A. Potter, and P. A. Campbell, "*Listeria monocytogenes* infects human endothelial cells by two distinct mechanisms," *Infection and Immunity*, vol. 63, no. 11, pp. 4268–4276, 1995.

[229] D. A. Drevets, "Dissemination of *Listeria monocytogenes* by infected phagocytes," *Infection and Immunity*, vol. 67, no. 7, pp. 3512–3517, 1999.

[230] S. L. Wilson and D. A. Drevets, "*Listeria monocytogenes* infection and activation of human brain microvascular endothelial cells," *Journal of Infectious Diseases*, vol. 178, no. 6, pp. 1658–1666, 1998.

[231] D. A. Drevets, T. A. Jelinek, and N. E. Freitag, "*Listeria monocytogenes*-infected phagocytes can initiate central nervous system infection in mice," *Infection and Immunity*, vol. 69, no. 3, pp. 1344–1350, 2001.

[232] L. Greiffenberg, W. Goebel, K. S. Kim, et al., "Interaction of *Listeria monocytogenes* with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells," *Infection and Immunity*, vol. 66, no. 11, pp. 5260–5267, 1998.

[233] T. J. Abbruscato and T. P. Davis, "Protein expression of brain endothelial cell E-cadherin after hypoxia/aglycemia: influence of astrocyte contact," *Brain Research*, vol. 842, no. 2, pp. 277–286, 1999.

[234] D. Pal, K. L. Audus, and T. J. Siahaan, "Modulation of cellular adhesion in bovine brain microvessel endothelial cells by a decapeptide," *Brain Research*, vol. 747, no. 1, pp. 103–113, 1997.

[235] J. Szmydynger-Chodobska, C. L. Pascale, A. N. Pfeffer, C. Coulter, and A. Chodobski, "Expression of junctional proteins in choroid plexus epithelial cell lines: a comparative study," *Cerebrospinal Fluid Research*, vol. 4, article 11, 2007.

[236] N. Krueger, C. Low, and W. Donachie, "Phenotypic characterization of the cells of the inflammatory response in ovine encephalitic listeriosis," *Journal of Comparative Pathology*, vol. 113, no. 3, pp. 263–275, 1995.

[237] E. A. Antal, E. M. Løberg, P. Bracht, K. K. Melby, and J. Mæhlen, "Evidence for intraaxonal spread of *Listeria monocytogenes* from the periphery to the central nervous system," *Brain Pathology*, vol. 11, no. 4, pp. 432–438, 2001.

[238] E. Domann, M. Deckert, D. Schlüter, and T. Chakraborty, "*Listeria monocytogenes*: a model system to study invasion and spread of bacteria in the central nervous system," *Current Topics in Microbiology and Immunology*, vol. 265, pp. 213–226, 2002.

[239] L. Dons, K. Weclewicz, Y. Jin, E. Bindseil, J. E. Olsen, and K. Kristensson, "Rat dorsal root ganglia neurons as a model for *Listeria monocytogenes* infections in culture," *Medical Microbiology and Immunology*, vol. 188, no. 1, pp. 15–21, 1999.

[240] L. Dons, Y. Jin, K. Kristensson, and M. E. Rottenberg, "Axonal transport of *Listeria monocytogenes* and nerve-cell-induced bacterial killing," *Journal of Neuroscience Research*, vol. 85, no. 12, pp. 2529–2537, 2007.

[241] Y. Jin, L. Dons, K. Kristensson, and M. E. Rottenberg, "Neural route of cerebral *Listeria monocytogenes* murine infection:

role of immune response mechanisms in controling bacterial neuroinvasion," *Infection and Immunity*, vol. 69, no. 2, pp. 1093–1100, 2001.

[242] Y. Jin, G. Lundkvist, L. Dons, K. Kristensson, and M. E. Rottenberg, "Interferon-$\gamma$ mediates neuronal killing of intracellular bacteria," *Scandinavian Journal of Immunology*, vol. 60, no. 5, pp. 437–448, 2004.

[243] O. F. Join-Lambert, S. Ezine, A. Le Monnier, et al., "*Listeria monocytogenes*-infected bone marrow myeloid cells promote bacterial invasion of the central nervous system," *Cellular Microbiology*, vol. 7, no. 2, pp. 167–180, 2005.

[244] J. Altimira, N. Prats, S. López, et al., "Repeated oral dosing with *Listeria monocytogenes* in mice as a model of central nervous system listeriosis in man," *Journal of Comparative Pathology*, vol. 121, no. 2, pp. 117–125, 1999.

[245] D. Schlüter, S. B. Oprisiu, S. Chahoud, et al., "Systemic immunization induces protective $CD4^+$ and $CD8^+$ T cell-mediated immune responses in murine *Listeria monocytogenes* meningoencephalitis," *European Journal of Immunology*, vol. 25, no. 8, pp. 2384–2391, 1995.

[246] D. Schlüter, S. Chahoud, H. Lassmann, A. Schumann, H. Hof, and M. Deckert-Schlüter, "Intracerebral targets and immunomodulation of murine *Listeria monocytogenes* meningoencephalitis," *Journal of Neuropathology and Experimental Neurology*, vol. 55, no. 1, pp. 14–24, 1996.

[247] D. Schlüter, C. Buck, S. Reiter, et al., "Immune reactions to *Listeria monocytogenes* in the brain," *Immunobiology*, vol. 201, no. 2, pp. 188–195, 1999.

[248] M. Deckert, S. Soltek, G. Geginat, et al., "Endogenous interleukin-10 is required for prevention of a hyperinflammatory intracerebral immune response in *Listeria monocytogenes* meningoencephalitis," *Infection and Immunity*, vol. 69, no. 7, pp. 4561–4571, 2001.

[249] L.-Y. Kwok, H. Miletic, S. Lütjen, et al., "Protective immunosurveillance of the central nervous system by *Listeria*-specific CD4 and CD8 T cells in systemic listeriosis in the absence of intracerebral *Listeria*," *Journal of Immunology*, vol. 169, no. 4, pp. 2010–2019, 2002.

[250] S. Virna, M. Deckert, S. Lütjen, et al., "TNF is important for pathogen control and limits brain damage in murine cerebral listeriosis," *Journal of Immunology*, vol. 177, no. 6, pp. 3972–3982, 2006.

[251] M. Deckert, S. Virna, M. Sakowicz-Burkiewicz, et al., "Interleukin-1 receptor type 1 is essential for control of cerebral but not systemic listeriosis," *American Journal of Pathology*, vol. 170, no. 3, pp. 990–1002, 2007.

[252] D. L. Montgomery and R. W. Storts, "*Listeria monocytogenes* meningoencephalitis: murine models vs. the naturally occurring disease in ruminants," *Journal of Neuropathology and Experimental Neurology*, vol. 55, no. 5, p. 603, 1996.

[253] M. Peters and M. Hewicker-Trautwein, "Studies on the cell tropism of *Listeria monocytogenes* in ovine fetal brain cell cultures," *Veterinary Microbiology*, vol. 49, no. 3-4, pp. 169–179, 1996.

[254] D. Urbaneck, "Zur Neuropathogenese der Listeriose. Histologische Untersuchungen am N. trigeminus bei spontan an zerebraler Form der Listeriose erkrankten Schafen," *Archiv für Experimentelle Veterinärmedizin*, vol. 16, pp. 61–80, 1962.

[255] O. Asahi, T. Hosoda, and Y. Akiyama, "Studies on the relationship between neuropenetrability of *L. monocytogenes* and the mechanisms of immunity acquired by subcutaneous infection with its live culture," *The Journal of Veterinary Medical Science*, vol. 17, supplement, p. 17, 1955.

[256] B. J. Currie, D. A. Fisher, D. M. Howard, and J. N. C. Burrow, "Neurological melioidosis," *Acta Tropica*, vol. 74, no. 2-3, pp. 145–151, 2000.

[257] A. C. Cheng and B. J. Currie, "Melioidosis: epidemiology, pathophysiology, and management," *Clinical Microbiology Reviews*, vol. 18, no. 2, pp. 383–416, 2005.

[258] P. W. Ladds, A. D. Thomas, and B. Pott, "Melioidosis with acute meningoencephalomyelitis in a horse," *Australian Veterinary Journal*, vol. 57, no. 1, pp. 36–38, 1981.

[259] K. T. Wong, S. D. Puthucheary, and J. Vadivelu, "The histopathology of human melioidosis," *Histopathology*, vol. 26, no. 1, pp. 51–55, 1995.

[260] J. M. Stevens, E. E. Galyov, and M. P. Stevens, "Actin-dependent movement of bacterial pathogens," *Nature Reviews Microbiology*, vol. 4, no. 2, pp. 91–101, 2006.

[261] W. J. Wiersinga, T. van der Poll, N. J. White, N. P. Day, and S. J. Peacock, "Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*," *Nature Reviews Microbiology*, vol. 4, no. 4, pp. 272–282, 2006.

[262] C. Olson, C. L. Rollins, V. Bagdonas, I. C. Blore, and D. Segre, "Distribution of *Listeria monocytogenes* in listeriosis of sheep," *The Journal of Infectious Diseases*, vol. 93, no. 3, pp. 247–256, 1953.

[263] J. W. Osebold and T. Inouye, "Pathogenesis of *Listeria monocytogenes* infections in natural hosts. II. Sheep studies," *The Journal of Infectious Diseases*, vol. 95, no. 1, pp. 67–78, 1954.

[264] W. Jakob, "Weitere experimentelle untersuchungen zur pathogenese der zerebralen Listeriose des Schafes. I. Mitteilung: pathologisch-anatomische Befunde nach experimenteller Infektion mit frisch isolierten Listerienstämmen," *Archiv für Experimentelle Veterinarmedizin*, vol. 20, no. 2, pp. 367–381, 1966.

[265] J. C. Low and W. Donachie, "Clinical and serum antibody responses of lambs to infection by *Listeria monocytogenes*," *Research in Veterinary Science*, vol. 51, no. 2, pp. 185–192, 1991.

[266] A. Miettinen, J. Husu, and J. Tuomi, "Serum antibody response to *Listeria monocytogenes*, listerial excretion, and clinical characteristics in experimentally infected goats," *Journal of Clinical Microbiology*, vol. 28, no. 2, pp. 340–343, 1990.

[267] J. Schleicher and D. Urbaneck, "Ein beitrag zur experimentellen Listeriose bei Haus- und versuchstieren. X. Mitteilung: experimentell erzeugte zerebrale Listeriose beim schaf durch neurale und paraneurale Injektionen mit geringen kulturdosen an zweigen des nervus trigeminus," *Archiv für Experimentelle Veterinarmedizin*, vol. 20, no. 1, pp. 23–48, 1966.

[268] D. Urbaneck, "Ein beitrag zur experimentellen Listeriose bei Haus- und versuchstieren. III. Mitteilung: versuche zur Klärung der neuropathogenese der zentralnervösen Listerioseform bei schafen durch applikation von listerien am N. trigeminus," *Archiv für Experimentelle Veterinarmedizin*, vol. 16, pp. 641–677, 1962.

[269] G. Borman, C. Olson, and D. Segre, "The trigeminal and facial nerves as pathways for infection of sheep with *Listeria monocytogenes*," *American Journal of Veterinary Research*, vol. 21, pp. 993–1000, 1960.

[270] S. Blanot, M. M. Joly, F. Vilde, et al., "A gerbil model for rhombencephalitis due to *Listeria monocytogenes*," *Microbial Pathogenesis*, vol. 23, no. 1, pp. 39–48, 1997.

[271] O. Disson, G. Nikitas, S. Grayo, et al., "Modeling human listeriosis in natural and genetically engineered animals," *Nature Protocols*, vol. 4, no. 6, pp. 799–810, 2009.

[272] Y. Jin, L. Dons, K. Kristensson, and M. E. Rottenberg, "Colony-stimulating factor 1-dependent cells protect against systemic infection with *Listeria monocytogenes* but facilitate neuroinvasion," *Infection and Immunity*, vol. 70, no. 8, pp. 4682–4686, 2002.

[273] M. Takeichi, H. Inuzuka, K. Shimamura, M. Matsunaga, and A. Nose, "Cadherin-mediated cell-cell adhesion and neurogenesis," *Neuroscience Research*, vol. 13, supplement, pp. S92–S96, 1990.

[274] K. Shimamura and M. Takeichi, "Local and transient expression of E-cadherin involved in mouse embryonic brain morphogenesis," *Development*, vol. 116, no. 4, pp. 1011–1019, 1992.

[275] K. Shimamura, T. Takahashi, and M. Takeichi, "E-cadherin expression in a particular subset of sensory neurons," *Developmental Biology*, vol. 152, no. 2, pp. 242–254, 1992.

[276] S. Dramsi, S. Lévi, A. Triller, and P. Cossart, "Entry of *Listeria monocytogenes* into neurons occurs by cell-to-cell spread: an in vitro study," *Infection and Immunity*, vol. 66, no. 9, pp. 4461–4468, 1998.

[277] M. Doumith, C. Cazalet, N. Simoes, et al., "New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays," *Infection and Immunity*, vol. 72, no. 2, pp. 1072–1083, 2004.

[278] D. E. Conner, V. N. Scott, S. S. Summer, and D. T. Bernard, "Pathogenicity of food-borne, environmental and clinical isolates of *Listeria monocytogenes* in mice," *Journal of Food Protection*, vol. 54, pp. 1553–1556, 1989.

[279] J. McLauchlin, "Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 9, no. 3, pp. 210–213, 1990.

[280] R. Brosch, B. Catimel, G. Milton, et al., "Virulence heterogeneity of *Listeria monocytogenes* strains from various sources (human food animal) in immunocompetent mice and its associated typing characteristics," *Journal of Food Protection*, vol. 56, pp. 296–301, 1993.

[281] J. McLauchlin, "The pathogenicity of *Listeria monocytogenes*: a public health perspective," *Reviews in Medical Microbiology*, vol. 8, no. 1, pp. 1–14, 1997.

[282] M. Wiedmann, J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough, and C. A. Batt, "Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential," *Infection and Immunity*, vol. 65, no. 7, pp. 2707–2716, 1997.

[283] M. Wiedmann, "Molecular subtyping methods for *Listeria monocytogenes*," *Journal of AOAC International*, vol. 85, no. 2, pp. 524–531, 2002.

[284] J. W. Davies, E. P. Ewan, P. Varughese, and S. E. Acres, "*Listeria monocytogenes* infections in Canada," *Clinical and Investigative Medicine*, vol. 7, no. 4, pp. 315–320, 1984.

[285] D. Art and P. André, "Clinical and epidemiological aspects of Listeriosis in Belgium, 1985–1990," *Zentralblatt für Bakteriologie*, vol. 275, no. 4, pp. 549–556, 1991.

[286] A. Schuchat, B. Swaminathan, and C. V. Broome, "Epidemiology of human listeriosis," *Clinical Microbiology Reviews*, vol. 4, no. 2, pp. 169–183, 1991.

[287] C. Jacquet, E. Gouin, D. Jeannel, P. Cossart, and J. Rocourt, "Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin," *Applied and Environmental Microbiology*, vol. 68, no. 2, pp. 616–622, 2002.

[288] P. Gracieux, S. M. Roche, P. Pardon, and P. Velge, "Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid' L. mono media," *International Journal of Food Microbiology*, vol. 83, no. 2, pp. 133–145, 2003.

[289] S. Kathariou, "*Listeria monocytogenes* virulence and pathogenicity, a food safety perspective," *Journal of Food Protection*, vol. 65, no. 11, pp. 1811–1829, 2002.

[290] D. R. Fenlon, T. Stewart, and W. Donachie, "The incidence, numbers and types of *Listeria monocytogenes* isolated from farm bulk tank milks," *Letters in Applied Microbiology*, vol. 20, no. 1, pp. 57–60, 1995.

[291] N. E. Jensen, F. M. Aarestrup, J. Jensen, and H. C. Wegener, "*Listeria monocytogenes* in bovine mastitis. Possible implication for human health," *International Journal of Food Microbiology*, vol. 32, no. 1-2, pp. 209–216, 1996.

[292] E. T. Ryser, S. M. Arimi, M. M. Bunduki, and C. W. Donnelly, "Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media," *Applied and Environmental Microbiology*, vol. 62, no. 5, pp. 1781–1787, 1996.

[293] D. J. Trott, I. D. Robertson, and D. J. Hampson, "Genetic characterisation of isolates of *Listeria monocytogenes* from man, animals and food," *Journal of Medical Microbiology*, vol. 38, no. 2, pp. 122–128, 1993.

[294] W. F. Bibb, B. G. Gellin, R. Weaver, et al., "Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations," *Applied and Environmental Microbiology*, vol. 56, no. 7, pp. 2133–2141, 1990.

[295] C. Buchrieser, R. Brosch, B. Catimel, and J. Rocourt, "Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks," *Canadian Journal of Microbiology*, vol. 39, no. 4, pp. 395–401, 1993.

[296] C. Jacquet, B. Catimel, R. Brosch, et al., "Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992," *Applied and Environmental Microbiology*, vol. 61, no. 6, pp. 2242–2246, 1995.

[297] J.-C. Piffaretti, H. Kressebuch, M. Aeschbacher, et al., "Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 10, pp. 3818–3822, 1989.

[298] D. P. Ewert, L. Lieb, P. S. Hayes, M. W. Reeves, and L. Mascola, "*Listeria monocytogenes* infection and serotype distribution among HIV- infected persons in Los Angeles County, 1985–1992," *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology*, vol. 8, no. 5, pp. 461–465, 1995.

[299] J. Nolla-Salas, J. Bosch, I. Gasser, et al., "Perinatal listeriosis: a population based multicenter study in Barcelona, Spain (1990–1996)," *American Journal of Perinatology*, vol. 15, no. 8, pp. 461–467, 1998.

[300] B. Swaminathan and P. Gerner-Smidt, "The epidemiology of human listeriosis," *Microbes and Infection*, vol. 9, no. 10, pp. 1236–1243, 2007.

[301] S. Larsson, "Epidemiology of listeriosis in Sweden 1958–1974," *Scandinavian Journal of Infectious Diseases*, vol. 11, no. 1, pp. 47–54, 1979.

[302] P. Gerner-Smidt, S. Ethelberg, P. Schiellerup, et al., "Invasive listeriosis in Denmark 1994–2003: a review of 299 cases

with special emphasis on risk factors for mortality," *Clinical Microbiology and Infection*, vol. 11, no. 8, pp. 618–624, 2005.

[303] O. F. Rasmussen, P. Skouboe, L. Dons, L. Rossen, and J. E. Olsen, "*Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes," *Microbiology*, vol. 141, no. 9, pp. 2053–2061, 1995.

[304] G. T. Jeffers, J. L. Bruce, P. L. McDonough, et al., "Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases," *Microbiology*, vol. 147, no. 5, pp. 1095–1104, 2001.

[305] K. K. Nightingale, K. Windham, and M. Wiedmann, "Evolution and molecular phylogeny of *Listeria monocytogenes* isolated from human and animal listeriosis cases and foods," *Journal of Bacteriology*, vol. 187, no. 16, pp. 5537–5551, 2005.

[306] B. Jeršek, P. Gilot, M. Gubina, et al., "Typing of *Listeria monocytogenes* strains by repetitive element sequence-based PCR," *Journal of Clinical Microbiology*, vol. 37, no. 1, pp. 103–109, 1999.

[307] B. Nørrung and J. K. Andersen, "Variations in virulence between different electrophoretic types of *Listeria monocytogenes*," *Letters in Applied Microbiology*, vol. 30, no. 3, pp. 228–232, 2000.

[308] S. M. Roche, P. Velge, E. Bottreau, et al., "Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice," *International Journal of Food Microbiology*, vol. 68, no. 1-2, pp. 33–44, 2001.

[309] L. Pine, S. Kathariou, F. Quinn, et al., "Cytopathogenic effects in enterocytelike Caco-2 cells differentiate virulent from avirulent *Listeria* strains," *Journal of Clinical Microbiology*, vol. 29, no. 5, pp. 990–996, 1991.

[310] M. Tabouret, J. De Rycke, A. Audurier, and B. Poutrel, "Pathogenicity of *Listeria monocytogenes* isolates in immunocompromised mice in relation to listeriolysin production," *Journal of Medical Microbiology*, vol. 34, no. 1, pp. 13–18, 1991.

[311] N. Van Langendonck, E. Bottreau, S. Bailly, et al., "Tissue culture assays using Caco-2 cell line differentiate virulent from non-virulent *Listeria monocytogenes* strains," *Journal of Applied Microbiology*, vol. 85, no. 2, pp. 337–346, 1998.

[312] K. K. Nightingale, R. A. Ivy, A. J. Ho, et al., "InlA premature stop codons are common among *Listeria monocytogenes* isolates from foods and yield virulence-attenuated strains that confer protection against fully virulent strains," *Applied and Environmental Microbiology*, vol. 74, no. 21, pp. 6570–6583, 2008.

[313] R. Jonquieres, H. Bierne, J. Mengaud, and P. Cossart, "The *inlA* gene of *Listeria monocytogenes* LO28 harbors a nonsense mutation resulting in release of internalin," *Infection and Immunity*, vol. 66, no. 7, pp. 3420–3422, 1998.

[314] A. Roberts, Y. Chan, and M. Wiedmann, "Definition of genetically distinct attenuation mechanisms in naturally virulence-attenuated *Listeria monocytogenes* by comparative cell culture and molecular characterization," *Applied and Environmental Microbiology*, vol. 71, no. 7, pp. 3900–3910, 2005.

[315] S. M. Roche, P. Gracieux, E. Milohanic, et al., "Investigation of specific substitutions in virulence genes characterizing phenotypic groups of low-virulence field strains of *Listeria monocytogenes*," *Applied and Environmental Microbiology*, vol. 71, no. 10, pp. 6039–6048, 2005.

[316] P. Velge, M. Herler, J. Johansson, et al., "A naturally occurring mutation K220T in the pleiotropic activator PrfA of *Listeria monocytogenes* results in a loss of virulence due to decreasing DNA-binding affinity," *Microbiology*, vol. 153, part 4, pp. 995–1005, 2007.

[317] S. Témoin, S. M. Roche, O. Grépinet, Y. Fardini, and P. Velge, "Multiple point mutations in virulence genes explain the low virulence of *Listeria monocytogenes* field strains," *Microbiology*, vol. 154, no. 3, pp. 939–948, 2008.

[318] T. Nishibori, K. Cooray, H. Xiong, et al., "Correlation between the presence of virulence-associated genes as determined by PCR and actual virulence to mice in various strains of *Listeria* spp," *Microbiology and Immunology*, vol. 39, no. 5, pp. 345–349, 1995.

[319] Z. W. Jaradat, G. E. Schutze, and A. K. Bhunia, "Genetic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes," *International Journal of Food Microbiology*, vol. 76, no. 1-2, pp. 1–10, 2002.

[320] S. Chiu, P. B. Vanderlinde, and G. A. Dykes, "A comparison of selected methods for measuring the virulence properties of *Listeria* spp," *Canadian Journal of Microbiology*, vol. 52, no. 4, pp. 301–307, 2006.

[321] D. Liu, M. L. Lawrence, L. Gorski, et al., "*Listeria monocytogenes* serotype 4b strains belonging to lineages I and III possess distinct molecular features," *Journal of Clinical Microbiology*, vol. 44, no. 1, pp. 214–217, 2006.

[322] A. H. Barbour, A. Rampling, and C. E. Hormaeche, "Variation in the infectivity of *Listeria monocytogenes* isolates following intragastric inoculation of mice," *Infection and Immunity*, vol. 69, no. 7, pp. 4657–4660, 2001.

[323] A. M. Lammerding, K. A. Glass, A. Gendron-Fitzpatrick, and M. P. Doyle, "Determination of virulence of different strains of *Listeria monocytogenes* and *Listeria innocua* by oral inoculation of pregnant mice," *Applied and Environmental Microbiology*, vol. 58, no. 12, pp. 3991–4000, 1992.

[324] S. M. Roche, P. Gracieux, I. Albert, et al., "Experimental validation of low virulence in field strains of *Listeria monocytogenes*," *Infection and Immunity*, vol. 71, no. 6, pp. 3429–3436, 2003.

[325] S. M. Roche, A. Kerouanton, J. Minet, et al., "Prevalence of low-virulence *Listeria monocytogenes* strains from different foods and environments," *International Journal of Food Microbiology*, vol. 130, no. 2, pp. 151–155, 2009.

[326] A. Jensen, L. E. Thomsen, R. L. Jørgensen, et al., "Processing plant persistent strains of *Listeria monocytogenes* appear to have a lower virulence potential than clinical strains in selected virulence models," *International Journal of Food Microbiology*, vol. 123, no. 3, pp. 254–261, 2008.

[327] C. N. Larsen, B. Nørrung, H. M. Sommer, and M. Jakobsen, "In vitro and in vivo invasiveness of different pulsed-field gel electrophoresis types of *Listeria monocytogenes*," *Applied and Environmental Microbiology*, vol. 68, no. 11, pp. 5698–5703, 2002.

[328] S. H. Kim, M. K. Bakko, D. Knowles, and M. K. Borucki, "Oral inoculation of A/J mice for detection of invasiveness differences between *Listeria monocytogenes* epidemic and environmental strains," *Infection and Immunity*, vol. 72, no. 7, pp. 4318–4321, 2004.

[329] D. Liu, M. L. Lawrence, M. Wiedmann, et al., "*Listeria monocytogenes* subgroups IIIA, IIIB, and IIIC delineate genetically distinct populations with varied pathogenic potential," *Journal of Clinical Microbiology*, vol. 44, no. 11, pp. 4229–4233, 2006.

[330] D. Liu, M. L. Lawrence, A. J. Ainsworth, and F. W. Austin, "Toward an improved laboratory definition of *Listeria monocytogenes* virulence," *International Journal of Food Microbiology*, vol. 118, no. 2, pp. 101–115, 2007.

[331] S. Gudmundsdóttir, S. M. Roche, K. G. Kristinsson, and M. Kristjánsson, "Virulence of *Listeria monocytogenes* isolates from humans and smoked salmon, peeled shrimp, and their processing environments," *Journal of Food Protection*, vol. 69, no. 9, pp. 2157–2160, 2006.

[332] A. R. Kidd and S. Terlecki, "Visceral and cerebral listeriosis in a lamb," *Veterinary Record*, vol. 78, no. 13, pp. 453–454, 1966.

[333] A. H. Killinger and M. E. Mansfield, "Epizootiology of listeric infection in sheep," *Journal of the American Veterinary Medical Association*, vol. 157, no. 10, pp. 1318–1324, 1970.

[334] H. Behrens and A. Krüger, "Differentiation of listeria strains isolated in brain listeriosis of sheep," *Berliner und Münchener Tierarztliche Wochenschrift*, vol. 82, no. 1, pp. 4–6, 1969.

[335] D. Urbaneck, "Ein beitrag zur experimentellen listeriose bei Haus- und l aboratoriumstieren. IV. Mitteilung: versuche zur Klärung der zentralnervösen listerioseform bei schafen durch applikation von listerien im bereich der äusseren haut und verschiedener schleimhäute des Kopfes (paraneurale Infektion)," *Archiv für Experimentelle Veterinärmedizin*, vol. 17, pp. 35–53, 1963.

[336] G. Trautwein and H. Behrens, "Histopathologie der Meningoencephalitis listeriosa des Schafes," *Deutsche Tierarztliche Wochenschrift*, vol. 69, pp. 149–155, 1962.

[337] M. A. Pohl, M. Wiedmann, and K. K. Nightingale, "Associations among *Listeria monocytogenes* genotypes and distinct clinical manifestations of listeriosis in cattle," *American Journal of Veterinary Research*, vol. 67, no. 4, pp. 616–626, 2006.

[338] R. Nappi, E. Bozzetta, R. Serra, et al., "Molecular characterization of *Listeria monocytogenes* strains associated with outbreaks of listeriosis in humans and ruminants and food products by serotyping and automated ribotyping," *Veterinary Research Communications*, vol. 29, supplement 2, pp. 249–252, 2005.

[339] H. Unnerstad, A. Romell, H. Ericsson, M. L. Danielsson-Tham, and W. Tham, "*Listeria monocytogenes* in faeces from clinically healthy dairy cows in Sweden," *Acta Veterinaria Scandinavica*, vol. 41, no. 2, pp. 167–171, 2000.

[340] J. S. Van Kessel, J. S. Karns, L. Gorski, B. J. McCluskey, and M. L. Perdue, "Prevalence of *Salmonellae, Listeria monocytogenes*, and fecal coliforms in bulk tank milk on US dairies," *Journal of Dairy Science*, vol. 87, no. 9, pp. 2822–2830, 2004.

[341] J. R. Husu, J. T. Seppanen, S. K. Sivela, and A. L. Rauramaa, "Contamination of raw milk by *Listeria monocytogenes* on dairy farms," *Zentralblatt für Veterinarmedizin. Reihe B*, vol. 37, no. 4, pp. 268–275, 1990.

[342] M. K. Borucki, C. C. Gay, J. Reynolds, et al., "Genetic diversity of *Listeria monocytogenes* strains from a high-prevalence dairy farm," *Applied and Environmental Microbiology*, vol. 71, no. 10, pp. 5893–5899, 2005.

[343] L. Hassan, H. O. Mohammed, P. L. McDonough, and R. N. Gonzalez, "A cross-sectional study on the prevalence of *Listeria monocytogenes* and *Salmonella* in New York dairy herds," *Journal of Dairy Science*, vol. 83, no. 11, pp. 2441–2447, 2000.

[344] A. J. Ho, V. R. Lappi, and M. Wiedmann, "Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility," *Journal of Dairy Science*, vol. 90, no. 5, pp. 2517–2524, 2007.

[345] E. Garcia, M. de Paz, J. L. Rodriguez, et al., "Exogenous sources of *Listeria* contamination in raw ewe's milk," *Journal of Food Protection*, vol. 59, no. 9, pp. 950–954, 1996.

[346] J. I. Esteban, B. Oporto, G. Aduriz, R. A. Juste, and A. Hurtado, "Faecal shedding and strain diversity of *Listeria monocytogenes* in healthy ruminants and swine in Northern Spain," *BMC Veterinary Research*, vol. 5, article 2, 2009.

[347] E. Zundel and S. Bernard, "*Listeria monocytogenes* translocates throughout the digestive tract in asymptomatic sheep," *Journal of Medical Microbiology*, vol. 55, no. 12, pp. 1717–1723, 2006.

[348] W. Muraoka, C. Gay, D. Knowles, and M. Borucki, "Prevalence of *Listeria monocytogenes* subtypes in bulk milk of the Pacific Northwest," *Journal of Food Protection*, vol. 66, no. 8, pp. 1413–1419, 2003.

[349] T. Yoshida, M. Sato, and K. Hirai, "Prevalence of *Listeria* species in raw milk from farm bulk tanks in Nagano prefecture," *Journal of Veterinary Medical Science*, vol. 60, no. 3, pp. 311–314, 1998.

[350] E. Waak, W. Tham, and M. L. Danielsson-Tham, "Prevalence and fingerprinting of *Listeria monocytogenes* strains isolated from raw whole milk in farm bulk tanks and in dairy plant receiving tanks," *Applied and Environmental Microbiology*, vol. 68, no. 7, pp. 3366–3370, 2002.

[351] B. M. Jayarao, S. C. Donaldson, B. A. Straley, et al., "A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania," *Journal of Dairy Science*, vol. 89, no. 7, pp. 2451–2458, 2006.

[352] D. Schoder, P. Winter, A. Kareem, W. Baumgartner, and M. Wagner, "A case of sporadic ovine mastitis caused by *Listeria monocytogenes* and its effect on contamination of raw milk and raw-milk cheeses produced in the on-farm dairy," *Journal of Dairy Research*, vol. 70, no. 4, pp. 395–401, 2003.

[353] I. Eilertz, M. L. Danielsson-Tham, K. E. Hammarberg, et al., "Isolation of *Listeria monocytogenes* from goat cheese associated with a case of listeriosis in goat," *Acta Veterinaria Scandinavica*, vol. 34, no. 2, pp. 145–149, 1993.

[354] P. Winter, F. Schilcher, Z. Bagò, et al., "Clinical and histopathological aspects of naturally occurring mastitis caused by *Listeria monocytogenes* in cattle and ewes," *Journal of Veterinary Medicine Series B*, vol. 51, no. 4, pp. 176–179, 2004.

[355] C. M. B. S. Pintado, K. A. Grant, R. Halford-Maw, et al., "Association between a case study of asymptomatic ovine listerial mastitis and the contamination of soft cheese and cheese processing environment with *Listeria monocytogenes* in Portugal," *Foodborne Pathogens and Disease*, vol. 6, no. 5, pp. 569–575, 2009.

[356] L. M. Graves, S. B. Hunter, A. R. Ong, et al., "Microbiological aspects of the investigation that traced the 1998 outbreak of listeriosis in the United States to contaminated hot dogs and establishment of molecular subtyping-based surveillance for *Listeria monocytogenes* in the PulseNet network," *Journal of Clinical Microbiology*, vol. 43, no. 5, pp. 2350–2355, 2005.

[357] S. J. Olsen, M. Patrick, S. B. Hunter, et al., "Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat," *Clinical Infectious Diseases*, vol. 40, no. 7, pp. 962–967, 2005.

[358] P. S. Mead, E. F. Dunne, L. Graves, et al., "Nationwide outbreak of listeriosis due to contaminated meat," *Epidemiology and Infection*, vol. 134, no. 4, pp. 744–751, 2006.

[359] H. Ericsson, A. Eklöw, M.-L. Danielsson-Tham, et al., "An outbreak of listeriosis suspected to have been caused by

rainbow trout," *Journal of Clinical Microbiology*, vol. 35, no. 11, pp. 2904–2907, 1997.

[360] S.-I. Makino, K. Kawamoto, K. Takeshi, et al., "An outbreak of food-borne listeriosis due to cheese in Japan, during 2001," *International Journal of Food Microbiology*, vol. 104, no. 2, pp. 189–196, 2005.

[361] J. J. Carrique-Mas, I. Hökeberg, Y. Andersson, et al., "Febrile gastroenteritis after eating on-farm manufactured fresh cheese—an outbreak of listeriosis?" *Epidemiology and Infection*, vol. 130, no. 1, pp. 79–86, 2003.

[362] M. L. Danielsson-Tham, E. Eriksson, S. Helmersson, et al., "Causes behind a human cheese-borne outbreak of gastrointestinal listeriosis," *Foodborne Pathogens and Disease*, vol. 1, no. 3, pp. 153–159, 2004.

[363] P. D. M. MacDonald, R. E. Whitwam, J. D. Boggs, et al., "Outbreak of listeriosis among Mexican immigrants as a result of consumption of illicitly produced Mexican-style cheese," *Clinical Infectious Diseases*, vol. 40, no. 5, pp. 677–682, 2005.

[364] O. Tresse, K. Shannon, A. Pinon, et al., "Variable adhesion of *Listeria monocytogenes* isolates from food-processing facilities and clinical cases to inert surfaces," *Journal of Food Protection*, vol. 70, no. 7, pp. 1569–1578, 2007.

[365] J. M. Lunden, M. K. Miettinen, T. J. Autio, and H. J. Korkeala, "Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times," *Journal of Food Protection*, vol. 63, no. 9, pp. 1204–1207, 2000.

[366] S. M. Arimi, T. J. Pritchard, and C. W. Donnelly, "Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments," *Journal of Food Protection*, vol. 60, no. 7, pp. 811–816, 1997.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 747892, 16 pages
doi:10.1155/2010/747892

*Review Article*

# Neuroinvasion in Prion Diseases: The Roles of Ascending Neural Infection and Blood Dissemination

**Sílvia Sisó, Lorenzo González, and Martin Jeffrey**

*Veterinary Laboratories Agency (VLA-Lasswade), Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ, UK*

Correspondence should be addressed to Sílvia Sisó, s.siso@vla.defra.gsi.gov.uk

Received 30 October 2009; Accepted 8 March 2010

Academic Editor: Marylou V. Solbrig



Prion disorders are infectious, neurodegenerative diseases that affect humans and animals. Susceptibility to some prion diseases such as kuru or the new variant of Creutzfeldt-Jakob disease in humans and scrapie in sheep and goats is influenced by polymorphisms of the coding region of the prion protein gene, while other prion disorders such as fatal familial insomnia, familial Creutzfeldt-Jakob disease, or Gerstmann-Straussler-Scheinker disease in humans have an underlying inherited genetic basis. Several prion strains have been demonstrated experimentally in rodents and sheep. The progression and pathogenesis of disease is influenced by both genetic differences in the prion protein and prion strain. Some prion diseases only affect the central nervous system whereas others involve the peripheral organs prior to neuroinvasion. Many experiments undertaken in different species and using different prion strains have postulated common pathways of neuroinvasion. It is suggested that prions access the autonomic nerves innervating peripheral organs and tissues to finally reach the central nervous system. We review here published data supporting this view and additional data suggesting that neuroinvasion may concurrently or independently involve the blood vascular system.

## 1. Background

Other than by direct invasion following traumatic and iatrogenic incidents, infectious agents can gain access to the brain by two mechanisms: using peripheral nerves as physical conduits (neural neuroinvasion) or via the blood (haematogenous neuroinvasion). Documented examples of the first include viruses, such as lyssaviruses or herpesviruses, and some bacterial infections such as listeriosis. Examples of the second include lentiviruses or flaviviruses and meningitides and thromboembolic encephalitides of bacterial aetiology.

The precise nature of the infectious agent of the transmissible spongiform encephalopathies (TSEs) or prion diseases is still to be determined, although the most widely accepted theory (the "protein-only" hypothesis) is that they are caused by an infectious proteinaceous agent. This agent, usually termed "prion" and often designated as $PrP^{sc}$, hypothetically originates as a result of an aberrant misfolding process of the cellular, normal host prion protein ($PrP^{c}$). TSEs are a group of neurodegenerative disorders that can affect animals and humans and include, amongst others, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) in cervids, transmissible mink encephalopathy (TME), and Creutzfeldt-Jakob disease (CJD) and its variant form (vCJD) in humans. Common to other protein misfolding neurological disorders, like Alzheimer's disease, TSE-affected individuals progressively accumulate disease-associated abnormal forms of $PrP^{c}$, generically designated $PrP^{d}$, in the brain and, in many instances, in other tissues. Regardless of the precise nature of the infectious agent, and although TSEs can be experimentally or iatrogenically reproduced by a variety of routes, it is believed that most natural infections are acquired by the alimentary route, so that the first barrier that the infectious agent encounters is the gastrointestinal epithelium. Once this barrier has been crossed, TSE agents could theoretically reach the brain by the same two pathways as any other infectious agent.

In this review we will present the findings that have led to the prevailing hypothesis of neuroinvasion in animal TSEs, that is, the neural route. We will also discuss whether such findings and recent data are compatible with an alternative or complementary pathway, that is, the haematogenous route.

Emphasis will be laid on data from experimental TSE models in rodent species and sheep, although findings arising from natural infections in several species will also be considered.

One precaution to be taken when interpreting studies on pathogenesis is to consider carefully the techniques employed. In the case of TSEs, some of those studies, particularly early ones, were carried out by bioassay of selected tissues in laboratory animal species. While this approach is the only one that can demonstrate actual infectivity, it cannot determine which tissue structure or cell-type is infected because of the difficulties in fine dissection procedures and the risk of cross-contamination between tissues at necropsy. More recent and often comprehensive studies use $PrP^d$ as a surrogate marker of infection, albeit the correlation between the two is not always precise. The disease-specific form of the prion protein can be detected in the brain and viscera of TSE affected animals by a number of techniques, some of which, like ELISA or Western blot, employ extraction in detergents and incomplete enzymatic degradation to reveal protease-resistant PrP ($PrP^{res}$). However, these techniques still lack definition of the precise structures harbouring the surrogate marker because sample dissection is done on fresh or frozen tissue. $PrP^d$ detected by immunohistochemistry (IHC) circumvents issues of potential contamination and can be precisely ascribed to specific tissues, structures, and cells, and even to the intra- or extracellular compartments. Moreover, IHC is able to detect diverse morphological types of $PrP^d$ which, depending on the protocol used, can include both protease-resistant and protease-sensitive forms. By using a panel of PrP antibodies, the integrity of the protein (full length or truncated, and their location) can also be determined.

## 2. Neural Neuroinvasion: The Prevailing View

*2.1. Spread of Infectivity along Nerves.* A number of studies have provided evidence in support of anterograde and retrograde transport of prion infectivity along peripheral nerves, and of transynaptic spread of infectivity within the central nervous system (CNS). The most easily interpretable paradigms arose from experiments based on cranial and peripheral nerve challenges. Thus, intraocular injection results in spread of infectivity along retinal ganglion axons and once within the brain follows transynaptic dissemination through the visual pathways [1, 2]. Similarly, following intralingual inoculation, infectivity ascends rapidly and retrogradely via the XII cranial nerve reaching the hypoglossal nucleus within two weeks [3]. Curiously, evidence of transport along the olfactory nerves following intranasal exposure is still lacking [4, 5], and only the most recent experiments done by Bessen et al. [6] showed that only one hamster clinically affected out of five had traces of $PrP^d$ concomitantly within the olfactory sensory epithelium and in the glomerular layer of the olfactory bulbs. This result suggests that some transport of infectivity by olfactory nerves is possible although it is unclear if it would occur anterogradely from the CNS or retrogradely from the olfactory epithelium.

Estimates of the speed of axonal transport of prions vary from 0.5–3 mm/day along peripheral or cranial nerves, or within the central nervous system [7, 8]. Although these estimates face the significant confounding factor of the rate at which infectivity or $PrP^d$ is amplified or accumulated in the reporter cell or tissue, the data suggest that rates of spread of infectivity do not precisely correspond with either slow or fast axonal transport mechanisms. Nevertheless, neurotropic viruses also show a similar broad range of transport velocities; herpesviruses, for example, travel at rates of fast axonal transport [9], while the calculated velocity of rabies virus may be as low as 12 mm/day [10]. It is difficult therefore to conclude how prion infectivity may spread along neuronal processes simply from crude estimates of transport velocity.

The numerous intranerve transmission studies provide conclusive evidence that infectivity can be transported into the CNS along or within axons. There is also evidence that infection may also be transported out of the CNS along axons. Experimental rodent infections with the agents of scrapie and TME have shown that infection can spread from the thoracic segments of the spinal cord to their corresponding spinal [7] and to the sciatic [8] nerves. Similarly, intracerebral challenge of deer with cattle BSE results in $PrP^d$ in the enteric nervous system (ENS) in the absence of lymphoid tissue involvement and only after widespread $PrP^d$ accumulation in the brain [11]; such findings are most probably due to anterograde transport of infectivity from the CNS along nerve fibres.

*2.2. Involvement of Lymphoid Tissues and Peripheral Nerves in TSEs.* As early as 1967, studies of mouse scrapie after subcutaneous injection suggested that lymphoreticular system (LRS) tissues of hamsters such as the spleen and lymph nodes acquired infectivity prior to the brain [12]. In contrast, Kimberlin and Walker [13] did not find a similar role for the spleen when a different route was used. In those studies, intragastric administration of the scrapie strain 139A into mice concluded that agent replication in the Peyer's patches (PPs) occurred as fast as in the cervical lymph nodes, and earlier than in the spleen and CNS. Furthermore, splenectomy in those animals had no effect on incubation periods, in contrast to previous observations obtained after intraperitoneal inoculation with the same scrapie strain [14, 15]. These results, obtained in murine models, formed the pillars of the current neuroinvasion dogma, which suggests that after intraperitoneal infection neuroinvasion is initiated from the spleen and involves the sympathetic nervous system [14, 15], whereas neuroinvasion after intragastric infection is initiated directly from the intestines (via the PPs) and involves the enteric nervous system (ENS; [13]).

Several time course studies were initiated to investigate the spread of infectivity from the LRS to the CNS, [14, 16, 17]. Such studies reported a common neuroinvasion pathway for the intraperitoneal, intravenous (tail vein), and subcutaneous routes (scruff of the neck and foot pad), suggesting that neuroinvasion occurred through the spleen (and presumably visceral lymph nodes), along autonomic

nerves such as the splanchnic nerves to the mid-thoracic spinal cord, from where it would disseminated gradually to the rest of the CNS (brain and lumbar spinal cord). Although direct evidence for the initial spleen-thoracic spinal cord pathway was lacking because of difficulties in sampling small autonomic nerves and ganglia in mice, indirect evidence based on detection of infectivity in the stromal fraction of the spleen, in which nerve endings are abundant, was provided [18]. At the same time, centrifugal spread from the spinal cord and brain to peripheral nerves was demonstrated [7, 19].

Working with orally or intraperitoneally inoculated hamsters, Baldauf et al. [20] provided strong evidence for an alternative route of access of the 263 K strain to the brain that bypassed the spinal cord. It was proposed that the fibres of the vagus nerve would be the most likely direct pathway, although other cranial nerves and a blood- or even a cerebrospinal fluid- (CSF-) borne access were not ruled out. Similar to previous data obtained by Western blot analysis [21], spleens of infected hamsters did not consistently show $PrP^{res}$ thus questioning mandatory lymphoid tissue replication prior to neuroinvasion. Further evidence from experimental scrapie rodent models [22–26] confirmed the early involvement of the enteric and abdominal ganglia not only by doing infectivity assays but also by detecting the $PrP^{d}$ by IHC or paraffin-embedded tissue blot. Spatio-temporal studies on $PrP^{d}$ deposition revealed that the dorsal motor nucleus of the vagus nerve (DMNV) and the intermediolateral columns (IMLC) of the thoracic spinal cord were the first two CNS target sites to accumulate $PrP^{d}$. These authors suggested that the infectious agent reached the CNS retrogradely from the ENS by two different circuits: the splanchnic nerves circuit and the vagus nerve circuit as illustrated in Figure 1. The splanchnic circuit involved the cranial mesenteric and celiac ganglia and the IMLC followed by the dorsal root ganglia. The vagus nerve circuit involved the DMNV by passing the nodose ganglion. Subsequent experiments demonstrated the presence of infectivity in the vagus nerve and the cranial mesenteric ganglia by hamster bioassays [22].

Using 263 K infection of hamsters, Diringer [27] described a low level of "viraemia" lasting for at least 40 days, with detectable amounts of infectivity in the CNS, purportedly in capillary endothelial cells, but no sustained replication elsewhere. Experiments using the same hamster scrapie model discouraged consideration of haematogenous dissemination of infectivity by describing replication in the spleen and thoracic spinal cord earlier than in the brain [14, 28]. Evidence for a high and persistent "viraemia" was only available for some specific TSE models, such as the K.Fu strain of CJD in mice [29]. Similar to experiments in mice, the topographical $PrP^{d}$ distribution using the hamster model of 263 K did not reveal any evidence for haematogenous spread of infection to the brain [22]. Consequently, neuroinvasion in most studied rodent TSE models was thought to be crucially dependent on a non-blood-related compartment connecting the lymphoid tissue and the CNS, that is, the peripheral nervous system (PNS).

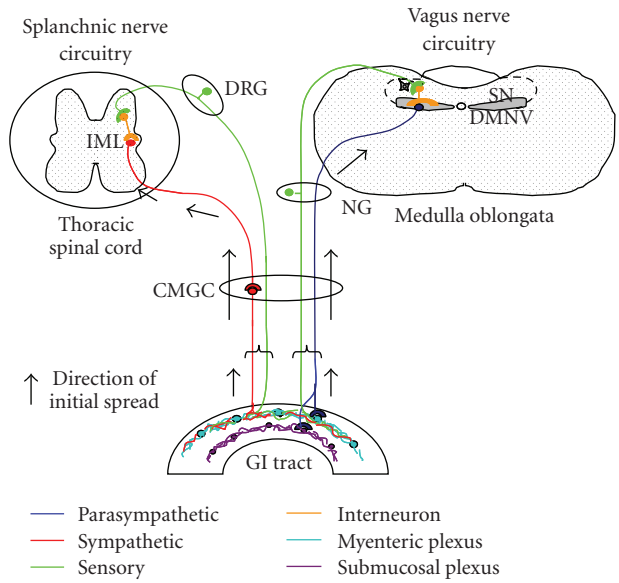



Figure 1: Schematic representation of the most generally accepted hypothesis of the routes of neuroinvasion in TSEs. Permission was obtained for reproducing this figure originally published in [22]. DRG, dorsal root ganglia; NG, nodose ganglion; CMGC, celiac and mesenteric ganglion complex; GI tract, gastrointestinal tract.

*2.3. Support to Neural Neuroinvasion Gained from Transgenic and Mutant Rodent Models.* An important contribution towards determining neuroinvasion pathways has been the use of transgenic, knockout, or mutant mice. From the early 1990s, such approaches have emphasised the role of key immune cell components in the up-take and replication of infectivity in the periphery and their contribution in transporting infectivity to the CNS [26]. In this respect, Klein et al. [30, 31] found no effect on susceptibility to disease or on spleen infectivity when mice deficient in T-lymphocytes were challenged with scrapie. In contrast, neuroinvasion was impaired in mice that were immunodeficient only in B-lymphocytes. Further investigations using scrapie-infected $TNF\alpha^{-/-}$ mice lacking mature follicular dendritic cells (FDCs) but not B lymphocytes [32, 33] concluded that accumulation of infectivity and $PrP^{res}/PrP^{d}$ in spleen and subsequent neuroinvasion were dependent upon mature FDCs. Parallel research towards investigating the role of macrophages in TSE pathogenesis concluded that macrophages were involved in the clearance of infectivity [34]. Complementary studies using transgenic mice overexpressing $PrP^{c}$ highlighted the importance of determining PrP interacting molecules or receptors which might be crucial for the normal function of FDCs and the interaction with B cells [26]. Although extensive research has focused on FDCs as cells for prion replication, current research highlights the role of dendritic cells, B-cells and macrophages in the transfer of infectivity from the gut to lymphoid organs [35, 36].

It is the interpolations between data obtained from all different animal models that have led to the most accepted view on absorption and transport of the infectious agent to the autonomic nervous system after oral exposure to

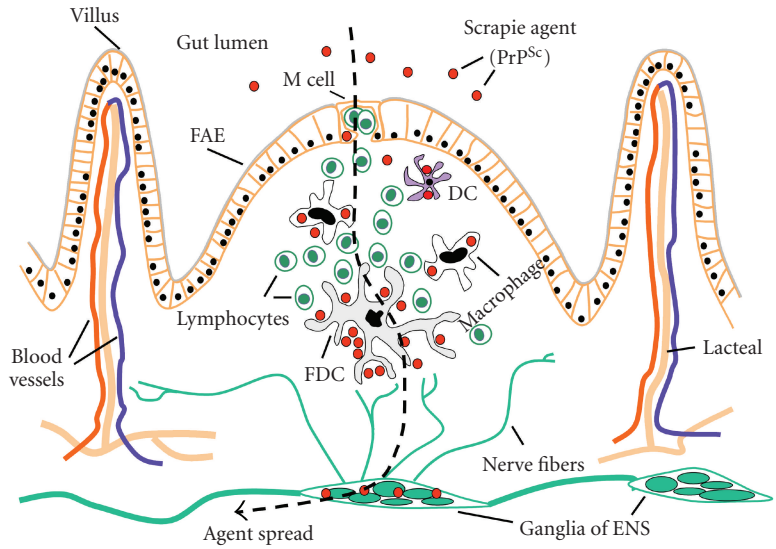



Figure 2: Diagrammatic representation of the possible pathways of absorption of TSE agents from the gut lumen and their transport to the ENS. Permission was obtained for reproducing this figure originally published in [26].

prions. Figure 2 provides a diagrammatic representation of the possible ways by which the ENS can become infected after oral/intragastric exposure. Many studies have focused on the role of M cells [25], dendritic cells [37], and possibly enteroendocrine cells as important candidates for the uptake or absorption of infectivity from the gastrointestinal tract. The infectious agent would then be transported to the lymphoid follicles of the PPs, where it would replicate in a B-cell and FDC-dependent manner. Subsequently, infectivity would reach nerve endings in and around the follicles and retrograde transport of the infectious agent would occur from the nerve endings towards the neuronal somata of the submucosal and myenteric plexuses. Alternatively, the agent could be transported from the gut lumen through the absorptive epithelium of the villi and could infect nerve endings of the lamina propria before being transported to the enteric plexuses [39].

Further support for the role of enteric neurons in the disease is their high $PrP^c$ mRNA levels [40]. In parallel, several rodent models have been produced in order to further support the idea that sympathetic nerves are responsible for transporting infectivity from the spleen to the spinal cord. Apparently this transfer of infectivity, which is facilitated in highly innervated spleens (as reviewed by [41]), occurs in a $PrP^c$-dependent fashion [42] and its rate is limited by the splenic sympathetic innervation [43]. The fact that mice with hyperinnervated spleens showed shorter incubation periods and higher infectivity titres and $PrP^d$ accumulation [43, 44] supports this statement, whereas the fact that denervated mice still develop scrapie [41] and, similarly, the failure of splenectomy to prevent disease implies that alternative routes participate in the neuroinvasion process and that spleen innervation might not be a prerequisite. In this respect, a major role of parasympathetic nerve fibers like the vagus nerve has been suspected for decades. However, similar evidence supporting the role of the vagus nerve in the pathogenesis of prion diseases is lacking as data from vagotomised rodent models is not available.

*2.4. Neuroinvasion in Experimental Sheep Models.* The first studies on sheep scrapie pathogenesis date back to the late 70s, at which period bioassay in rodents was the only tool available to test for the presence of infectivity in non-CNS tissues. Using such tools Hadlow et al. [45, 46] demonstrated that scrapie infectivity replicates in the PPs and other secondary lymphoid tissues before reaching the CNS. Interestingly, blood was postulated as the vehicle of infection for those peripheral LRS tissues, even though no infectivity was detected in blood from naturally scrapie-infected sheep at the time. Later, sheep experiments traced infectivity by $PrP^d$ or $PrP^{res}$ detection, [47–51] implicating, in addition to the PPs and other LRS tissues, the PNS, and the ENS in the spread of infectivity to the CNS.

Cell types supporting infectivity and $PrP^d$ accumulation are generally found throughout all lymphoid tissues, with the exception of the thymus, and usually also in neurons of the ENS [49, 53–55]. $PrP^d$ accumulation in LRS tissues is found in association with mature FDCs of the light zone and tingible body macrophages (TBMs) of the light and dark zones of secondary follicles (Figure 3). Ultrastructural studies confirmed that $PrP^d$ within the germinal centre of follicles locates mainly in association with the plasma membrane of FDCs and lysosomes of TBMs [56]. At early stages of infection, $PrP^d$ has been associated with dendritic cells or macrophages in the dome of the PPs in addition to cells of lymphoid tissues [53] or in the lacteals [38, 39]. The demonstration of initial $PrP^d$ accumulation in the gut-associated lymphoid tissue (GALT) and tonsil [49, 53] of sheep with preclinical natural scrapie strongly supported the hypothesis of infectivity crossing the mucosal barrier of the gastrointestinal tract by M-cell transcytosis followed by dendritic cell/macrophage transport, as illustrated in Figure 2.

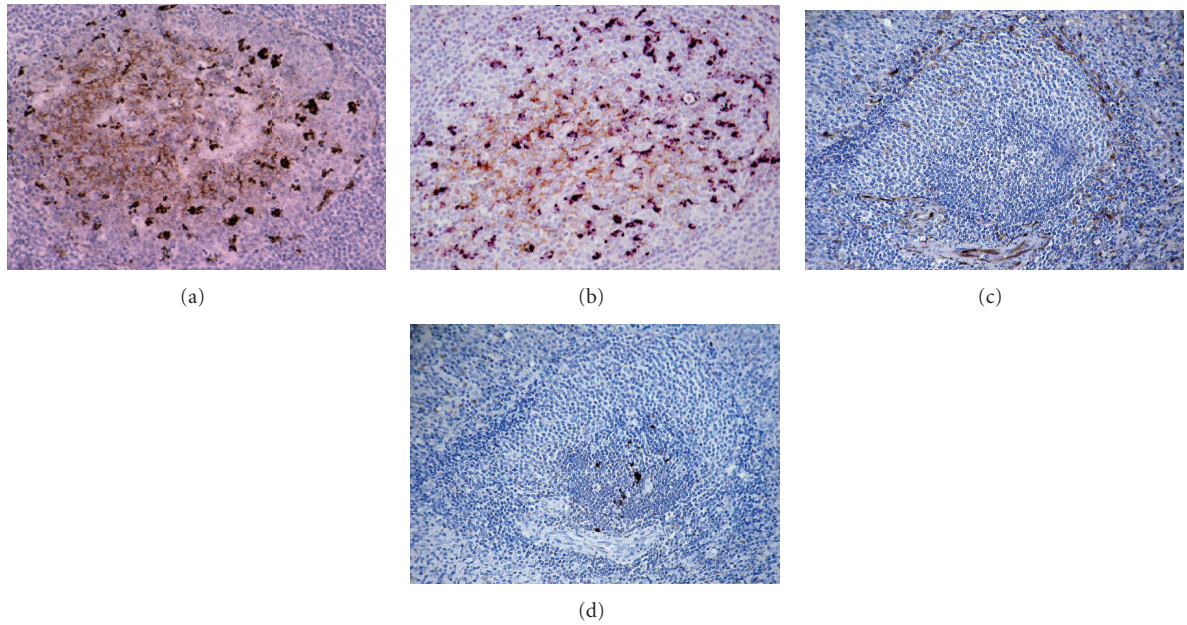



Figure 3: Scrapie infection in lymphoreticular tissues in ARQ/ARQ Suffolk sheep. (a) $PrP^d$ accumulation is associated with both FDC and macrophages in clinically affected sheep as shown by single IHC for PrP antibody R145. Peyer's patches of the distal ileum. (b) $PrP^d$ within TBMs is N terminally truncated whereas $PrP^d$ associated with FDC is not [52]. TBM and FDC can be distinguished in tissue sections by using N or C terminal PrP antibodies. FDCs (brown), labelled by the N terminal antibody FH11, are in light zone whereas TBMs (purple), labelled by the C terminal antibody R145, are both in dark and light zones of secondary lymphoid follicles: double IHC for FH11 (brown, DAB substrate) and R145 (purple, VIP substrate). In spleen, nerve endings located in the marginal zone ((c), IHC for PgP9.5) do not colocalised with $PrP^d$ deposition ((d), IHC for R145). (a)–(d) x20.

In a series of experiments, van Keulen and colleagues described the temporal spread of $PrP^d$ accumulation, in the GALT, non-GALT LRS, ENS, PNS, and CNS. They postulated that scrapie pathogenesis occurred in three different phases (Figure 4). In the first phase, after oral exposure to infection, the scrapie agent would be taken up by M cells followed by trancytosis to dendritic cells or macrophages, which would transport infection towards the secondary lymphoid follicles of the PPs and then drained to regional lymph nodes. A second phase of lymphatic dissemination would take place once infectivity is circulating the lymph (and blood) and therefore non-GALT LRS tissues would become involved. The fact that most data available on LRS examinations in sheep scrapie models describe infectivity of all lymphoid tissues at more or less the same time is highly suggestive of blood being an early contributing factor to dissemination of infection. Finally, neuroinvasion would arise in a third phase as a result of infection of nerve endings in the gut, GALT, and non-GALT LRS tissues and its retrograde spread through sympathetic and parasympathetic pathways.

Several sheep studies, encompassing sheep of different PrP genotypes, agree on the ENS being the first neural tissue in which $PrP^d$ can be demonstrated [49, 51, 57, 58]. In the latter study, the authors identified the duodenum and ileum as the initial ENS sites to accumulate $PrP^d$ and suggested subsequent spread towards the upper and lower gastrointestinal tract [51]. Infection of the different plexuses of the ENS could arise from various origins ([59]; reviewed by [60]): (i) fine nerve fibres underneath the villous epithelium could bring infection to the submucosal plexus; (ii) nerve endings at follicles of the PPs could also take infection to the submucosal plexus, (iii) indirect contact by means of lymph or active transport between the PPs and the ENS. In other studies, Heggebø et al. [59, 61] demonstrated $PrP^d$ accumulation in the marginal zone of the spleen, which is a highly innervated area. Therefore, as suggested for rodent models, retrograde transport from sympathetic nerve fibres connecting the spleen with the spinal cord could support neuroinvasion in sheep scrapie. Interpretation from all these studies postulated that entry to the DMNV was either by a retrograde spread of $PrP^d$ from the ENS along efferent parasympathetic fibres of the vagus nerve or by anterograde spread from other nuclei such as the nucleus of the tractus solitarii. The fact that neurons of the trigeminal and nodose ganglia accumulate $PrP^d$ later than the brain [51] would suggest that these ganglia become affected as a result of descending transport.

The interval between infection and first $PrP^d$ detection in peripheral tissues of naturally exposed sheep will depend on age of exposure and dose but experimental data show that strain and genotype also markedly influence rates of $PrP^d$ accumulation. Sheep of ARQ/ARQ genotype naturally infected with scrapie or experimentally infected with BSE [54, 55] accumulate $PrP^d$ in LRS tissues more slowly than do naturally or experimentally exposed VRQ/VRQ sheep [49, 53]. It is not clear whether these effects are solely strain or genotype effects or a combination of both.

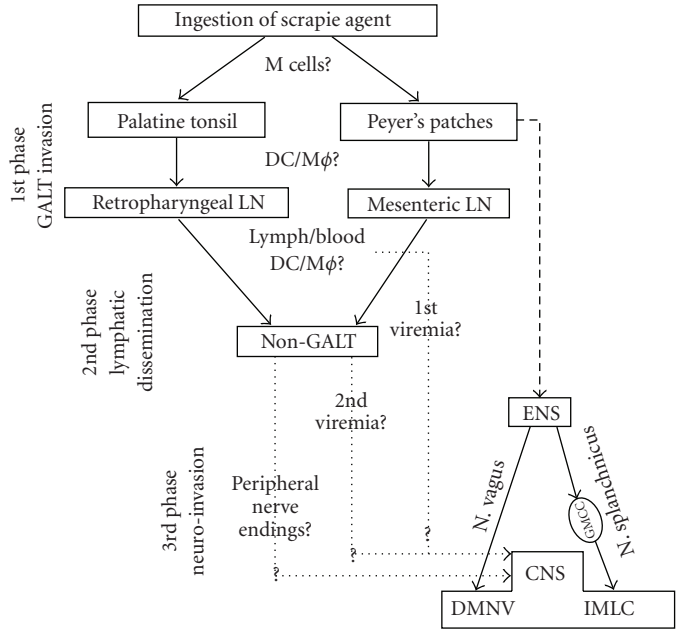



Figure 4: Pathogenesis of sheep scrapie. Permission was obtained for reproducing this figure originally published in [53]. For abbreviations see text.

Comprehensive pathogenetic studies have been performed on sheep infected with BSE, including infectivity assays [62] and tissue distribution of $PrP^d$ deposition by immunohistochemistry [55, 60, 62, 63]. Despite spatiotemporal differences reported between them, these studies complement each other as all had in common the type of inoculum, dose, and host *PRNP* genotype. Bellworthy et al. [62] demonstrated BSE infectivity in a wide range of tissues of ARQ/ARQ sheep including the PPs as early as 4 months postinfection, the spleen at 10 m after dosing, and other LRS tissues, CNS, and liver at 16 m postinoculation. In common with rodent studies, bioassay detecting infectivity was more sensitive than IHC in detecting presence of $PrP^d$. Resistance to oral BSE infection was observed in sheep of ARQ/ARR and ARR/ARR *PRNP* genotypes, which can be overcome if the intracerebral route is used [64], although the resulting magnitude of $PrP^d$ accumulation is lower than in ARQ/ARQ sheep inoculated by the same route [63], and LRS dissemination is only observed in sheep of the last genotype [65]. Importantly, no influence of the genotype or of the route of inoculation was observed on the signature of the BSE agent in terms of intracellular truncation of BSE $PrP^d$ and proteinase-K cleavage site of BSE $PrP^{res}$. These experiments suggest that the pathogenesis of experimental sheep BSE can differ depending on the route of inoculation and the host *PRNP* genotype.

Van Keulen et al. [60] showed the progressive accumulation of $PrP^d$ in 11 sheep orally dosed with cattle BSE and killed at 6 months postinfection and thereafter at 2-3-month interval. As with natural scrapie in VRQ/VRQ sheep, BSE-infected ARQ/ARQ Texel sheep developed clinical disease after peripheral accumulation of $PrP^d$ within the lymphoid tissues and autonomic nervous system. The first LRS tissues to accumulate $PrP^d$ were the GALT, followed by the GALT-draining lymph nodes and the spleen, and at a later stage, the non-GALT lymph nodes. Similar data were found in an earlier study albeit with some subtle differences. Jeffrey et al. [54] described first involvement of the retropharyngeal lymph nodes prior to the PPs in Romney sheep at 4 months post infection. Furthermore, $PrP^d$ deposition in CNS with no apparent LRS or PNS involvement up to 22 months postinoculation was observed in 40% (2/5) of clinically sick sheep [55]. In the Texel sheep, by 9 month postinoculation, $PrP^d$ was present in ENS neurons followed by the coeliac-mesenteric ganglion and the first two target sites in the CNS: the IMLC in the T7-L1 segments of the spinal cord and the DMNV in the brain. By 12-13 months dissemination to all non-GALT tissues had taken place. Colocalization of $PrP^d$ and CD68-positive macrophages was apparent in the marginal zone of spleen, suggesting a possible active trapping and phagocytosis of $PrP^d$ from circulating blood with potential for neuroinvasion as this is a compartment highly innervated by sympathetic autonomic fibres (Figure 3(c)). From 12-13 months postinoculation onwards spread within the ENS and IMLC affected all gastrointestinal tissues examined and all thoracic and lumbar spinal cord levels, respectively. It was not until 19 months postinoculation that sheep showed clinical signs of disease and $PrP^d$ deposition in the cerebral cortex.

*2.5. Neuroinvasion in Other Natural TSEs.* The mechanism of neuroinvasion in cattle affected with BSE remains uncertain. Although $PrP^d$ accumulation [66] and infectivity [66–70] has been reported in the GALT and ENS of cows orally challenged with exceedingly high doses of BSE, in natural BSE cases there is no known involvement of any viscera

or the PNS. A recent report of two animals that were part of a sequential kill experiment after oral exposure to a high dose of BSE agent concluded that BSE infectivity is retrogradely transported from the gastrointestinal tract to the CNS by two pathways [71]: (i) via the coeliac and mesenteric ganglion complex, through the splanchnic nerves towards the thoracolumbar spinal cord, and (ii) via the vagus nerve. Such conclusions arise from finding $PrP^d$ accumulation within the DMNV, coeliac and caudal mesenteric ganglia, and spinal cord segments but only in one of the reported cows. In this same experiment, authors anticipated that the presence of $PrP^d$ in the CNS prior to the dorsal root ganglia and possibly other peripheral nerves, that is, sciatic nerve, could be due to a secondary retrograde spread from the periphery to the CNS. Although the authors suggested a neural rather than lymphoreticular neuroinvasion-based pathway, importantly, none of the two cows had ENS involvement or showed $PrP^d$ in the vagus nerve even though several sections were examined.

The pathogenesis of CWD in cervids seems to vary between elk and deer [72]. Lymphoreticular dissemination occurs in preclinically affected deer but neuroinvasion has been reported in elk with no LRS involvement (reviewed by [73]). It is suspected that such differences might be partially defined by the species and the *PRNP* genotype. Accumulation of $PrP^d$ in the PNS has been demonstrated in mule deer with CWD [74], suggesting a similar pathogenesis to that reported for small ruminants with scrapie.

The mechanisms of neuroinvasion in transmissible mink encephalopathy (TME) have been extensively investigated in hamster models. Early studies described two biologically distinct strains in hamsters infected with TME, the hyper (HY) and the drowsy (DY) strains [75]. Both strains cause neuropathology in the CNS but only the HY disseminates within the LRS compartment. In a large number of experiments and using a variety of routes, Bartz et al. [3, 8, 76] have demonstrated that LRS involvement is not required for prion neuroinvasion, and that it occurs via peripheral or cranial nerves. Recent investigations [6] conclude that neuroinvasion is dependent on LRS involvement for specific routes of entry such as the nasal cavity but not for others such as the tongue (oral cavity). These different routes of ascending infection result in different CNS targeting [76].

For obvious reasons, the pathogenesis of CJD in the natural host is the least studied amongst prion diseases. The pathogenesis of sCJD differs from that of vCJD. Accumulation of $PrP^d$ is widespread in neural and lymphoreticular tissues in vCJD, whereas it is mostly restricted to the CNS in sCJD [77]. Consequently, tonsil and spleen biopsies are optimal tissues for preclinical vCJD diagnosis. Deposition of $PrP^d$ in the posterior roots of spinal cord with absence of ENS involvement in sCJD patients is thought to be due to centrifugal spread of infectivity [78]. Interestingly, some sCJD patients with prolonged clinical periods displayed detectable $PrP^{res}$ in skeletal-muscle and spleen homogenates by Western blot analysis [79]. Unfortunately, no IHC examination was performed in those positive samples. Thus $PrP^{res}$ deposition found in muscles could have been associated with peripheral nerves, muscle spindles, or myocytes and in spleen may have been associated with peripheral nerves or lymphoid follicles.

*2.6. Caveats in the Prevailing View of Neural Neuroinvasion.* Although the onset of $PrP^d$ accumulation in the LRS generally occurs significantly before that in the CNS, the consistency and time of appearance of such depositions varies depending on age, *PRNP* genotype, TSE strain, or source and route of inoculation. Thus, it is known that lymphoid follicles of the GALT undergo involution with age, and this is believed to have an effect on susceptibility to infection after oral exposure. In the case of TSEs, however, it has been shown that such physiological involution may be modulated by the infection itself, so that it does not seem to occur in the GALT that accumulates $PrP^d$ [66, 80, 81]. As far as the *PRNP* genotype is concerned, some earlier studies [82, 83] described lack of, or minimal, involvement of LRS tissues in sheep of the VRQ/ARR and VRQ/ARQ *PRNP* genotypes naturally infected with scrapie. Equally, a small proportion of scrapie-infected goats (4/72) were found to accumulate $PrP^d$ only in the brain and not in any of 10 LRS tissues examined; all those four animals carried the methionine allele at codon 142 [84]. Examples of the effect of the TSE agent strain or source include natural BSE [69], sporadic CJD [85], experimental CH1641 infection of sheep [38, 39], and Nor-98 [86], in all of which $PrP^d$ accumulates in the CNS without prior replication in LRS tissues. Therefore, to explain the neural neuroinvasion pathway, infection of the ENS and other nerve terminals of the autonomic nervous system would have to occur without previous LRS amplification.

Replication of infectivity in the GALT and non-GALT LRS tissues may often be demonstrated early in the disease process. However, nerve endings in such tissues may not necessarily become infected or if infected may not result in retrograde transport to the CNS. Peripheral nerve terminals can be found in the PPs of the intestine and could pick up infectivity by contact with $PrP^d$-containing TBMs and FDCs. However, while a rich network of nerves is present in the T-cell areas of the PPs—in which there is no $PrP^d$ accumulation—, nerves are very rarely found in the secondary follicles [59, 80, 87, 88], where FDCs, the principle cells which amplify infection in viscera, are located. Similarly, nerves cannot readily be detected in secondary follicles of other LRS tissues, particularly lymph nodes, and it is therefore difficult to see how infectivity could be transferred from the LRS to the PNS. Moreover, morphological analysis in confocal microscopy concluded that neuroimmune connections in PPs, spleens, and mesenteric lymph nodes from preclinical scrapie infected mice are established between dendritic cells in T-cell areas and peripheral nerves rather than arising from FDC in B-cell areas [36]. Although ovine noradrenergic fibres are relatively close to FDCs in the spleen [89, 90], no direct contact has been established, and patterns of immunolabelling for $PrP^d$ resembling nerve-like structures have not been reported. Innervation of lymphoid tissues is affected by the age-related involution process [91], so that a general increase in nerve density of the intestine

during early phases of life may contribute to an increased susceptibility of young animals to oral infection [80].

Following subcutaneous challenge, scrapie infectivity may reach the local lymph nodes via the afferent drainage and leave via efferent drainage to reach the blood. Recent studies done in sheep indicate that infectivity reaches the contra-lateral lymph nodes and lymph nodes distant to the site of inoculation almost as quickly as the ipsi-lateral nodes, suggesting that all of them were exposed more or less simultaneously (Chianini et al. unpublished observations). Similarly, naturally and orally challenged sheep with scrapie generally show early and widespread involvement of all LRS, including those that drain the head or limbs and are not anatomically linked to the alimentary system. The speed with which all lymph nodes become infected is not consistent with one lymph node or a group of lymph nodes being initially infected and subsequently amplifying infectivity for spread to other nodes.

The transcytosis and absorption of $PrP^d$ within a scrapie brain homogenate placed within the gut lumen was followed across the gut mucosa of sheep using IHC. $PrP^d$ within the homogenate was rapidly translocated (within 15 minutes) to mucosal and submucosal lymphatics [39]. From such a location infectivity would rapidly reach the vascular system. In this experiment no evidence to support transport of the homogenate across the dome and thus directly into PPs could be found; yet this model produced accelerated infection of PPs resulting in $PrP^d$ accumulation within 30 days, compared with more than 8 months for the corresponding natural disease. Thus, another possibility would be that germinal centres of LRS tissues are most exposed to infectivity via blood.

A recent study conducted on 67 preclinically infected sheep exposed to natural or experimental scrapie or BSE by various routes (oral, subcutaneous, intracerebral, or intravenous) showed that, regardless of the route, initial $PrP^d$ accumulation consistently occurred in the DMNV followed by the hypothalamus [92]. These findings are difficult to reconcile with a unique neuroinvasion ENS/PNS axis pathway. An alternative explanation would be that infection, which is present in blood, reaches the vagus nerve from its terminals in any organ or tissue compartment. Head et al. [93] have published the topographical brain distribution of amyloid plaques and lesion profiles of a linked human blood donor (orally infected) and blood recipient (intravenously infected). An almost identical severity and distribution of plaques and vacuoles were found both in donor and recipient thus supporting a common CNS portal of entry regardless of the route of exposure.

## 3. Haematogenous Neuroinvasion: The Alternative Hypothesis

*3.1. Evidence for Infectivity in the Blood.* Despite the fact that intravenous infection systematically resulted in shorter incubation periods than the intraperitoneal and subcutaneous routes, Kimberlin and Walker [16] were unable to find evidence of blood infectivity by bioassay. However, after further studies with the same 139A scrapie strain, a short "viraemic" phase immediately after injection was observed, leading Millson et al. [94] to conclude that the haematogenous spread of the agent was the likely means by which infection spread to the spleen and lymph nodes. Subsequent rodent blood bioassay experiments showed that a "viraemic" or "prionaemic" phase was evident early in the incubation period [27]. In the case of infection taking place by the oral route, it has been proposed that prionaemia could take place in two phases: an early one immediately after cell-mediated or cell-free uptake of infection from the gut lumen into the lymphatic system, and a second phase after amplification in LRS tissues [92].

An important series of experiments directed towards establishing the risk of transmission from blood products have provided the most comprehensive data on the presence of infectivity in blood. Infectivity demonstrated in the blood of BSE-infected sheep [92, 95, 96, 99], of natural scrapie cases [95, 100, 101], and of CWD-infected deer [102] agrees with data suggesting that vCJD can be transmitted by blood transfusion from donors incubating the disease. Descriptions of five transfusion-related vCJD cases have confirmed transmission of infection by blood-derived products [103–107]. Moreover, sheep experiments have shown that scrapie and BSE may be transmitted with blood collected early in the incubation period, even before any other evidence of infection is apparent [92, 95, 96], although the likelihood of transmission increased together with the incubation period in the blood donors. This would imply that infectivity in blood is an early and permanent (maybe even progressive) event rather than a transient one, in contradiction with the results on $PrP^{res}$ detection in the blood of hamsters by protein misfolding cyclic amplification [108].

*3.2. Evidence That Infectivity in Blood May Establish Tissue Infection.* Haematogenous dissemination of infection may have relevance for more than one aspect of TSE pathogenesis. Experiments with blood transfusions in sheep demonstrated how the route, oral (BSE donors) versus intravenous (BSE recipients) can affect peripheral pathogenesis (Figure 5). Although a similar scenario might be predicted for vCJD, it remains to be established if spleens of vCJD patients infected after receiving blood fractions contain higher $PrP^d$ magnitudes than those from patients infected by consumption of BSE-contaminated products. Accumulation of $PrP^d$ can be demonstrated in placenta [109], mammary gland [110], salivary glands [111], and kidney [96, 97, 112] of TSE-affected sheep. In the latter, with both scrapie [112] and BSE (S. Sisó, personal observations) infections, $PrP^d$ can be detected in mesenchymal cells between collecting ducts and loops of Henle in the renal pelvis in the absence of inflammation, suggesting that mesenchymal cells become infected from blood after filtration in the glomeruli or by extravasation from the vasa recta. Also in sheep, $PrP^d$ in the mammary gland [110, 113] and infectivity in milk [113, 114] also most probably derive from the blood, as it does in scrapie-affected goats, in which accumulation of $PrP^d$ in the mammary gland only occurred in animals with widespread

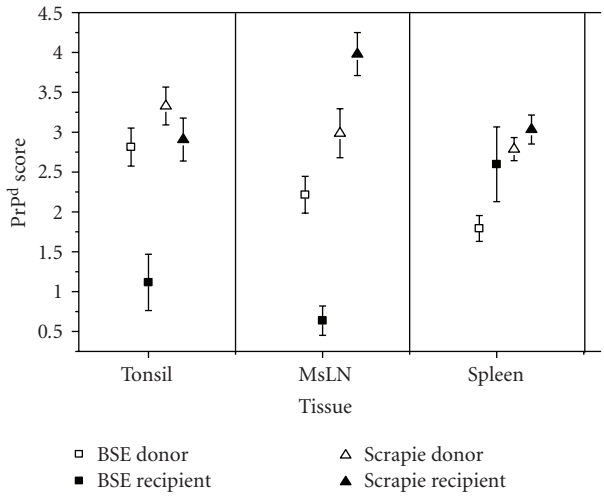



Figure 5: Magnitude of $PrP^d$ accumulation in LRS tissues of sheep as part of the blood transfusion experiment [95]. BSE donor sheep showed the highest levels of $PrP^d$ accumulation in palatine tonsil and mesenteric lymph node (MsLN) but the lowest in the spleen. In contrast, the spleen in BSE-transfused sheep showed significant higher magnitudes of $PrP^d$. Graphic representation including scrapie data has been modified from [96, 97].

LRS involvement regardless of the involvement or otherwise of neural tissues [115]. In the gut loop experiments described previously [39], in which $PrP^d$ was detected rapidly in lymphatics, PPs of the intestine became infected at an early disease stage: as no evidence was obtained for the inoculum being transported across the dome to the PPs, the most likely source of scrapie infection of FDCs is thought to be from blood.

*3.3. Evidence That Infectivity in Blood May Establish Brain Infection.* In the brains of BSE-infected sheep, the distribution of $PrP^d$ was found to be similar irrespective of the route of inoculation being intravascular, intracerebral, or alimentary [63], suggesting common patterns of neuroinvasion and CNS spread. The findings of vascular amyloid $PrP^d$ deposition in the basement membrane of endothelial cells of the hypothalamus [51] were an early indication that infectivity could reach the brain from the blood. However, such vascular $PrP^d$ plaques are a relatively rare finding in TSEs [116], which would not justify haematogenous neuroinvasion being a frequent event or having an important role.

It is generally accepted that access of infectious agents to the brain via the blood is hampered by a defensive mechanism: the so-called blood-brain barrier. However, there are specific structures in the brain, the circumventricular organs (CVOs) which, having fenestrated capillaries, are more permissive than other brain areas to the passage of large molecules and provide a two-way communication between CNS and the rest of the body. Recent studies have shown that $PrP^d$ accumulation in the CVOs of sheep was an early and consistent event that was not affected by the route of challenge or TSE strain, suggesting that CNS entry of infectivity can occur through these structures [92]. No evidence has been obtained that arrival of $PrP^d$ in the CVOs is cell-mediated, as white blood cells were not observed in these organs at preclinical or clinical stage of disease; it is therefore possible that TSE agents are present in plasma as cell-free, soluble molecules. This hypothesis would be reinforced by the above-mentioned findings of $PrP^d$ in kidneys of scrapie-affected sheep [96, 97, 112], which suggest that $PrP^d$ arrives in the renal papillary interstitium by filtration or extravasation of soluble molecules. Similarly, CVOs could initially up-take soluble infectivity present in plasma (or in CSF) and act as receptors, supporters, and transporters of infectivity. The variability in severity of $PrP^d$ accumulation observed between examined CVOs may suggest different participation of these organs in terms of acquiring and amplifying infectivity. Hypothetically, the sensory CVOs may be more susceptible to initial infection, and the secretory CVOs in releasing it to extracranial structures; that is, infectivity present in the pituitary gland may be transported into the adrenal gland.

Once infection is established in the sensory CVOs further spread to neighbouring neural structures through efferent connections could occur (Figure 6). Thus, for example, $PrP^d$ in the hypothalamic paraventricular nucleus and in the bed nucleus of the stria terminalis could result from spread from the subfornical organ and from the organum vasculosum of the lamina terminalis rather than from the DMNV. The involvement of the DMNV and the area postrema is often simultaneous [98, 115] making it difficult to determine if the mechanism of entry of infection at this level of the brain is through the vagus nerve or from the blood, or from both. If the access was from blood through the area postrema, or through CVOs in general, it is possible that neurons of these organs would be infected and would start to accumulate $PrP^d$ as soon as any circulating infectivity in blood reaches them. However, it might also be possible that neurons of neighbouring structures, such as the DMNV or the hypothalamus, to which CVOs are connected, amplify infectivity or accumulate $PrP^d$ more rapidly than CVO neurones, resulting in an earlier detection in those structures.

Until recently, the CVOs had received no or very little attention in any human or animal TSE. McBride et al. [22] reported absence of $PrP^d$ immunodeposits in the area postrema of hamsters experimentally inoculated with scrapie by the oral route but investigations did not address the involvement of other CVOs. Similarly, no CVOs were studied in mule deer with chronic wasting disease, in which the hypothalamus was reported to accumulate $PrP^d$ before the medulla but after the DMNV [117]. We routinely examine the CVOs in current experiments and have retrospectively examined formalin-fixed brains from earlier studies. So far, we have found $PrP^d$ deposition in CVOs from clinically affected animals of different species infected with several strains (Figure 7), such as in mice intracerebrally infected with ME7, 87V, 79A, 22A, and 301V strains, and deer [11] and cattle orally infected with cattle BSE, (Sisó et al. unpublished observations) and in goats with natural scrapie [115].

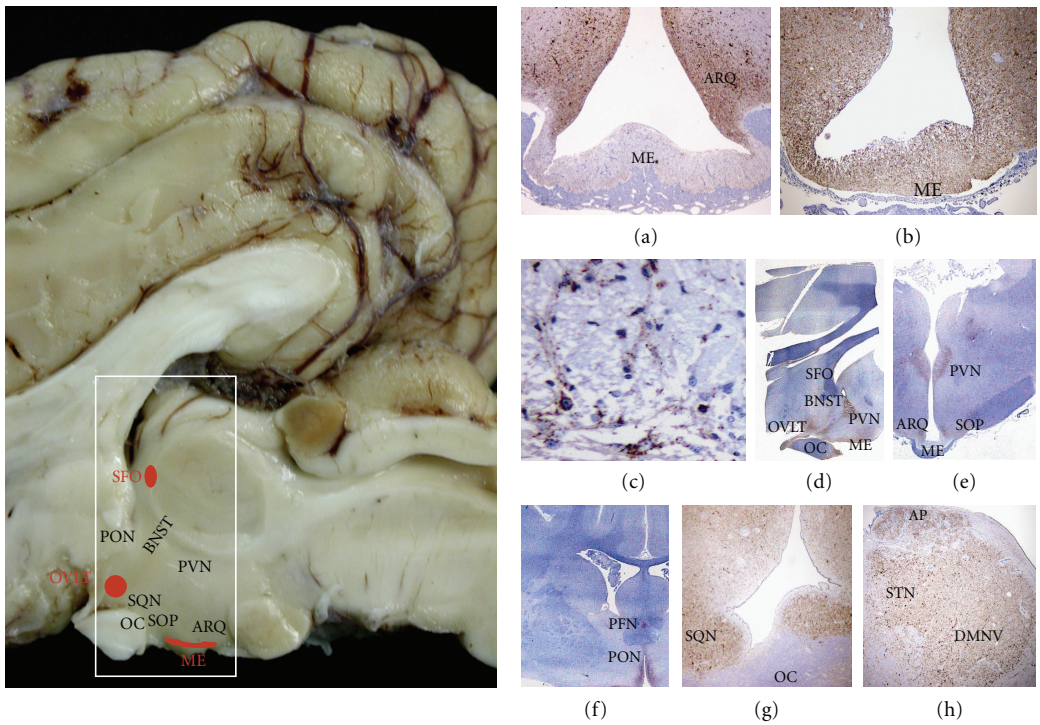



Figure 6: *CVO involvement may contribute to the spread of infection into the brain parenchyma.* Preclinically affected TSE sheep show mild early $PrP^d$ accumulation in the median eminence (ME) (a; x4), or severe deposition in later stages (b; x4). Higher magnification is needed to detect mild $PrP^d$ accumulation in the ME (c; x60). In later stages, preclinical sheep do show accumulation of $PrP^d$ in those brain areas with established connections with the CVOs. A sagittal section of the diencephalon (d; x1) has been produced from the area compressed in the white rectangle in the macroscopic sagittal view of the brain highlighting some of the neural structures. Thus, the involvement of the ME correlates with $PrP^d$ accumulation in the arcuate nucleus (ARQ) (a,b,e), and that of the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) correlates with $PrP^d$ in several nuclei: preoptic (PON) (f, x1), suprachiasmatic (SQN) (g; x4), supraoptic (SOP) (e, x1), paraventricular (PVN) (d, e), bed nucleus of the stria terminalis (BNST) (d) and perifornical (PFN) (f). Such correlations are difficult in the vagal complex because of the widespread severe $PrP^d$ accumulation (h).

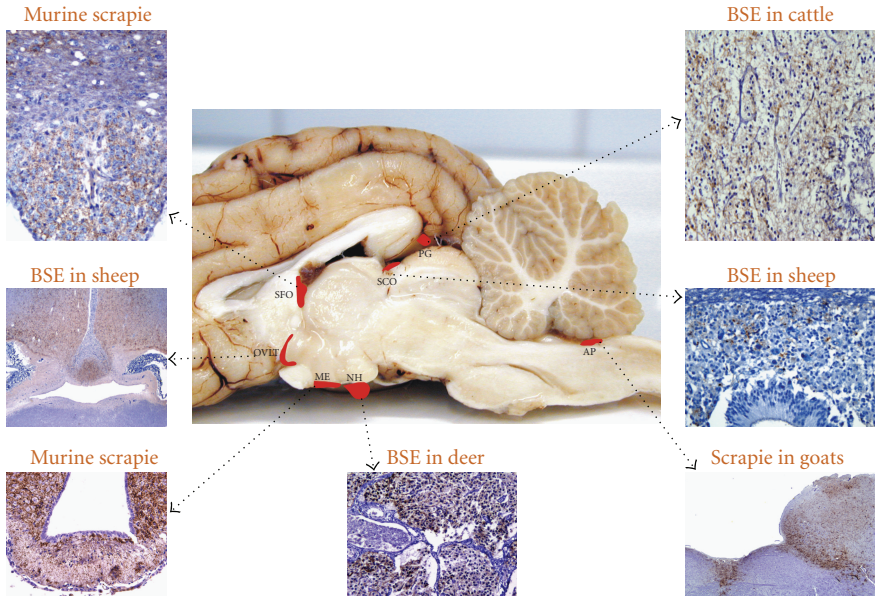



Figure 7: $PrP^d$ accumulation within CVOs in clinically affected animals with different TSE strains. AP, area postrema; PG, pineal gland; ME, median eminence; SCO, subcommisural organ; OVLT, organum vasculosum of lamina terminalis; SFO, subfornical organ; NH, neurophypophysis.

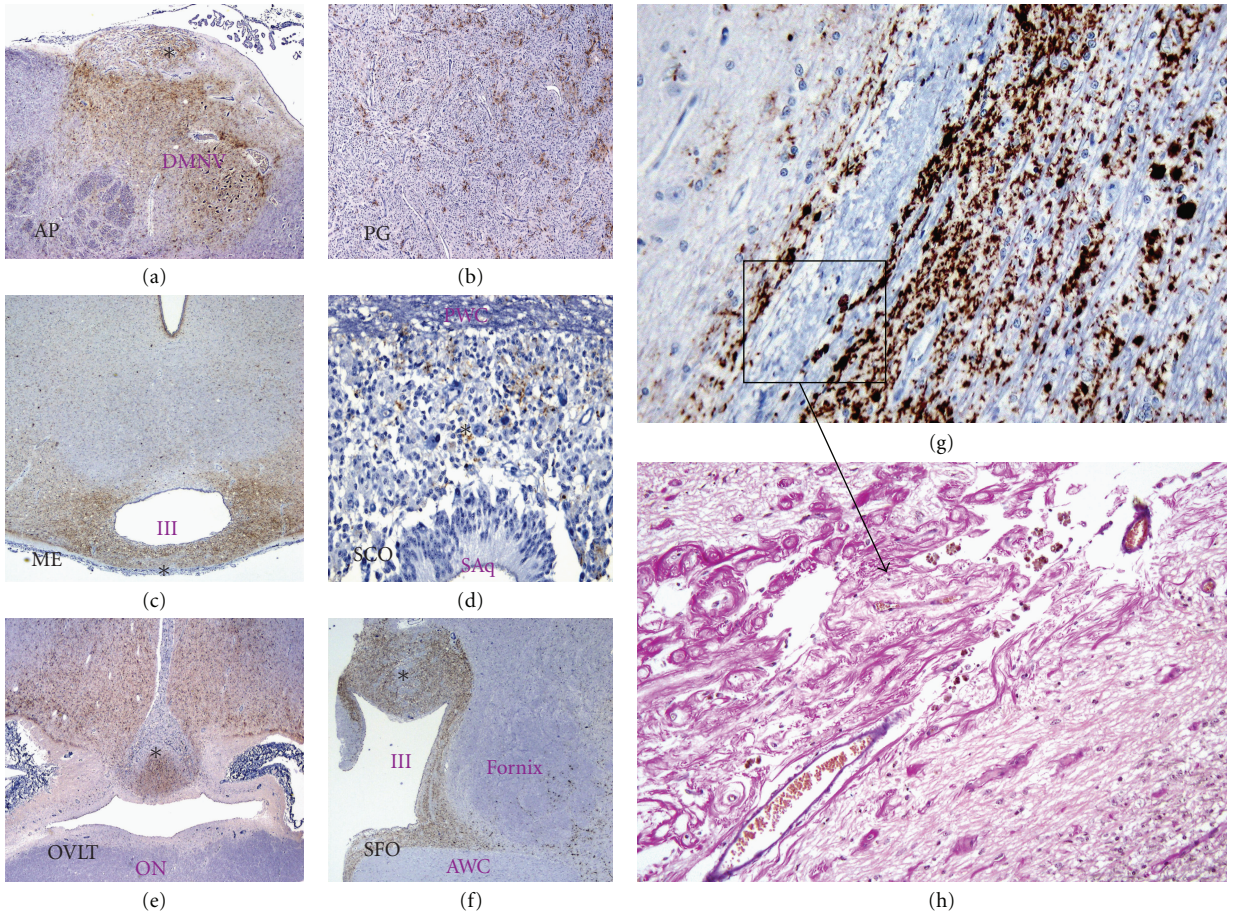



Figure 8: *PrP*$^{d}$ *accumulation in CVOs and in the porencephalic lesion*. Sheep CVOs highlighted with an asterix in pictures (a–f) of low magnification showed consistent PrP$^{d}$ deposition after intracerebral inoculation with BSE. Note that the porencephalic lesion which is surrounded by PrP$^{d}$ accumulations (g; x10) has abundant proliferation of newly formed vessels as revealed by a Van-Gieson staining (h; x20). CVOs: AP, area postrema; PG, pineal gland; ME, median eminence; SCO, subcommisural organ; OVLT, organum vasculosum of the lamina terminalis; SFO, subfornical organ. Brain structures: DMNV, dorsal nucleus of the vagus nerve; IV, fourth ventricle; Saq, Silvios' acqueduct; ON, optic chiasm; III, third ventricle; AWC, anterior white commisure. The figure is modified from (Sisó et al. [98]).

A further support for a blood-borne neuroinvasion pathway was provided by the study of the topographical distribution of PrP$^{d}$ in the brain of 27 sheep that had been intracerebrally challenged with BSE and presented porencephalic lesions resulting from the traumatic injury [98]. The key findings (Figure 8) were as follows: (i) same PrP$^{d}$ topography, involving bilaterally the DMNV and the hypothalamus, as for infections by other routes; (ii) involvement of CVOs; (iii) focal accumulation of PrP$^{d}$ at the porencephalic lesions, but extending locally to neighbouring ipsilateral cerebrocortical areas in some cases; and (iv) absence of PrP$^{d}$ in such lesions in cases that did not accumulate PrP$^{d}$ anywhere else in the brain. It was therefore suggested that, rather than a local replication of the infectious agent at the site of injection, the inoculum would be completely reabsorbed into the CSF and recirculated in the blood gaining access back to the brain through the CVOs and through the porencephalic lesions, where fenestrated neocapillaries were also abundant as part of the repair tissue process.

The haematogenous route, therefore, can represent a parallel or alternative pathway of neuroinvasion to ascending infection via the ENS and autonomic nervous system by accessing the CVOs and encephalic lesions of traumatic or vascular origin. Figure 9 represents schematically the possible pathways of neuroinvasion to be considered in TSEs.

## 4. Conclusions

The most widely accepted neuroinvasion mechanism in prion diseases is thought to involve amplification of infectivity within lymphoid tissues and subsequent retrograde spread of infection along autonomic nervous system nerves until it reaches the brain. However, the peripheral pathogenesis of prion diseases can vary depending on several factors. In most TSE cases, infection of the lymphoid system appears to facilitate neuroinvasion, increasing attack rates and shortening incubation periods, although in some scrapie cases and in most or all of natural BSE cases and in sCJD, the lymphoid system does not seem to be involved. In view

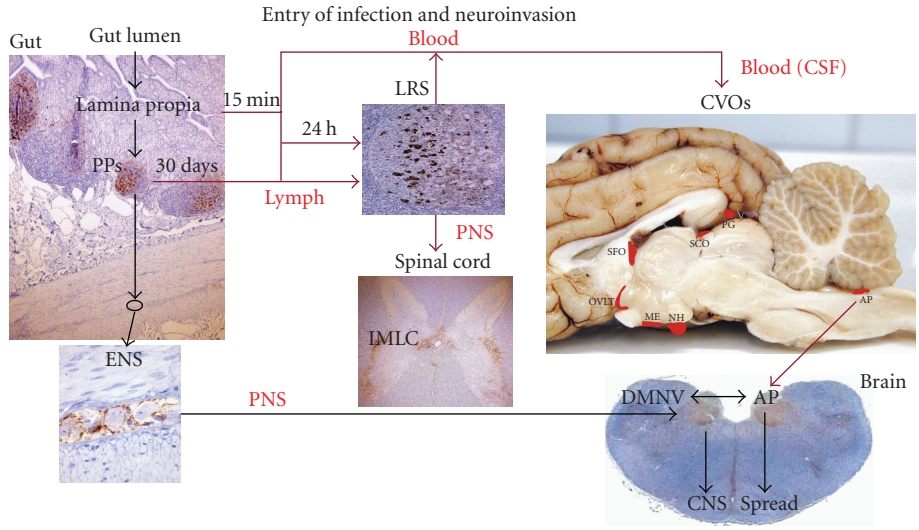



FIGURE 9: Schematic view of the neuroinvasion process involving the roles of the blood and the CVOs. For abbreviations see text and Figure 7 legend.

of the fact that, regardless of the route of infection and other factors that affect the peripheral pathogenesis (agent, *PRNP* genotype, etc.), the initial accumulation of $PrP^d$ in the brain and its spread appears to follow the same pattern, it is unlikely that neuroinvasion occurs solely by a mechanism involving retrograde transport of infectivity along peripheral nerves, at least those of the ENS/PNS axis, as the prevailing view suggests.

Alternatively, the observation that $PrP^d$ accumulates in peripheral organs, such as the kidney or the mammary gland, as does in the LRS tissues themselves, clearly shows a vascular dispersion of infectivity, which is consistent with an haematogenous route of neuroinvasion. Additional support for this alternative pathway derives from the early and systematic detection of $PrP^d$ in the CVOs which, lacking a blood-brain barrier, could act as portals of entry of infection.

Whether the haematogenous neuroinvasion pathway is coincidental with a neural pathway, and the relative importance of them for different TSEs, remains to be determined.

## References

[1] H. Fraser, "Neuronal spread of scrapie agent and targeting of lesions within the retino-tectal pathway," *Nature*, vol. 295, no. 5845, pp. 149–150, 1982.

[2] J. R. Scott and H. Fraser, "Transport and targeting of scrapie infectivity and pathology in the optic nerve projections following intraocular infection," *Progress in Clinical and Biological Research*, vol. 317, pp. 645–652, 1989.

[3] J. C. Bartz, A. E. Kincaid, and R. A. Bessen, "Rapid prion neuroinvasion following tongue infection," *Journal of Virology*, vol. 77, no. 1, pp. 583–591, 2003.

[4] A. E. Kincaid and J. C. Bartz, "The nasal cavity is a route for prion infection in hamsters," *Journal of Virology*, vol. 81, no. 9, pp. 4482–4491, 2007.

[5] M. Sbriccoli, F. Cardone, A. Valanzano, et al., "Neuroinvasion of the 263 K scrapie strain after intranasal administration occurs through olfactory-unrelated pathways," *Acta Neuropathologica*, vol. 117, no. 2, pp. 175–184, 2009.

[6] R. A. Bessen, S. Martinka, J. Kelly, and D. Gonzalez, "Role of the lymphoreticular system in prion neuroinvasion from the oral and nasal mucosa," *Journal of Virology*, vol. 83, no. 13, pp. 6435–6445, 2009.

[7] R. H. Kimberlin, H. J. Field, and C. A. Walker, "Pathogenesis of mouse scrapie: evidence for spread of infection from central to peripheral nervous system," *Journal of General Virology*, vol. 64, no. 3, pp. 713–716, 1983.

[8] J. C. Bartz, A. E. Kincaid, and R. A. Bessen, "Retrograde transport of transmissible mink encephalopathy within descending motor tracts," *Journal of Virology*, vol. 76, no. 11, pp. 5759–5768, 2002.

[9] M. L. Cook and J. G. Stevens, "Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection," *Infection and Immunity*, vol. 7, no. 2, pp. 272–288, 1973.

[10] P. Kucera, M. Dolivo, P. Coulon, and A. Flamand, "Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain," *Journal of Virology*, vol. 55, no. 1, pp. 158–162, 1985.

[11] S. Martin, M. Jeffrey, L. González, et al., "Immunohistochemical and biochemical characteristics of BSE and CWD in experimentally infected European red deer (Cervus elaphus elaphus)," *BMC Veterinary Research*, vol. 5, article 26, 2009.

[12] C. M. Eklund, R. C. Kennedy, and W. J. Hadlow, "Pathogenesis of scrapie virus infection in the mouse," *Journal of Infectious Diseases*, vol. 117, no. 1, pp. 15–22, 1967.

[13] R. H. Kimberlin and C. A. Walker, "Pathogenesis of scrapie in mice after intragastric infection," *Virus Research*, vol. 12, no. 3, pp. 213–220, 1989.

[14] R. H. Kimberlin and C. A. Walker, "Pathogenesis of experimental scrapie," in *Novel Infectious Agents and the Central Nervous System*, vol. 135 of *Ciba Foundation Symposium*, pp. 37–62, Wiley, Chichester, UK, 1988.

[15] R. H. Kimberlin and C. A. Walker, "The role of the spleen in the neuroinvasion of scrapie in mice," *Virus Research*, vol. 12, no. 3, pp. 201–212, 1989.

[16] R. H. Kimberlin and C. A. Walker, "Pathogenesis of mouse scrapie: effect of route of inoculation on infectivity titres and dose-response curves," *Journal of Comparative Pathology*, vol. 88, no. 1, pp. 39–47, 1978.

[17] R. H. Kimberlin and C. A. Walker, "Pathogenesis of mouse scrapie: patterns of agent replication in different parts of the CNS following intraperitoneal infection," *Journal of the Royal Society of Medicine*, vol. 75, no. 8, pp. 618–624, 1982.

[18] M. C. Clarke and R. H. Kimberlin, "Pathogenesis of mouse scrapie: distribution of agent in the pulp and stroma of infected spleens," *Veterinary Microbiology*, vol. 9, no. 3, pp. 215–225, 1984.

[19] N. Buyukmihci, F. Goehring-Harmon, and R. F. Marsh, "Asymmetry of retinal lesions in experimental scrapie after intracerebral inoculation of hamsters," *Experimental Neurology*, vol. 87, no. 1, pp. 172–176, 1985.

[20] E. Baldauf, M. Beekes, and H. Diringer, "Evidence for an alternative direct route of access for the scrapie agent to the brain bypassing the spinal cord," *Journal of General Virology*, vol. 78, no. 5, pp. 1187–1197, 1997.

[21] M. Beekes, E. Baldauf, and H. Diringer, "Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie," *Journal of General Virology*, vol. 77, no. 8, pp. 1925–1934, 1996.

[22] P. A. McBride, W. J. Schulz-Schaeffer, M. Donaldson, et al., "Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves," *Journal of Virology*, vol. 75, no. 19, pp. 9320–9327, 2001.

[23] M. Beekes, P. A. McBride, and E. Baldauf, "Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie," *Journal of General Virology*, vol. 79, no. 3, pp. 601–607, 1998.

[24] P. A. McBride and M. Beekes, "Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie," *Neuroscience Letters*, vol. 265, no. 2, pp. 135–138, 1999.

[25] M. Beekes and P. A. McBride, "Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie," *Neuroscience Letters*, vol. 278, no. 3, pp. 181–184, 2000.

[26] N. A. Mabbott and M. E. Bruce, "The immunology of TSE diseases," *Journal of General Virology*, vol. 82, pp. 2307–2318, 2001.

[27] H. Diringer, "Sustained viremia in experimental hamster scrapie. Brief report," *Archives of Virology*, vol. 82, no. 1-2, pp. 105–109, 1984.

[28] R. H. Kimberlin and C. A. Walker, "Pathogenesis of scrapie (strain 263k) in hamsters infected intracerebrally, intraperitoneally or intraocularly," *Journal of General Virology*, vol. 67, no. 2, pp. 255–263, 1986.

[29] Y. Kuroda, C. J. Gibbs Jr., H. L. Amyx, and D. C. Gajdusek, "Creutzfeldt-Jakob disease in mice: persistent viremia and preferential replication of virus in low-density lymphocytes," *Infection and Immunity*, vol. 41, no. 1, pp. 154–161, 1983.

[30] M. A. Klein, R. Frigg, E. Flechsig, et al., "A crucial role for B cells in neuroinvasive scraple," *Nature*, vol. 390, no. 6661, pp. 687–690, 1997.

[31] M. A. Klein, R. Frigg, A. J. Raeber, et al., "PrP expression in B lymphocytes is not required for prion neuroinvasion," *Nature Medicine*, vol. 4, no. 12, pp. 1429–1433, 1998.

[32] K. L. Brown, K. Stewart, D. L. Ritchie, et al., "Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells," *Nature Medicine*, vol. 5, no. 11, pp. 1308–1312, 1999.

[33] N. A. Mabbott, A. Williams, C. F. Farquhar, M. Pasparakis, G. Kollias, and M. E. Bruce, "Tumor necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie," *Journal of Virology*, vol. 74, no. 7, pp. 3338–3344, 2000.

[34] V. Beringue, M. Demoy, C. I. Lasmézas, et al., "Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis," *Journal of Pathology*, vol. 190, no. 4, pp. 495–502, 2000.

[35] G. A. Davies, A. R. Bryant, J. D. Reynolds, F. R. Jirik, and K. A. Sharkey, "Prion diseases and gastrointestinal tract," *Canadian Journal of Gastroenterology*, vol. 20, no. 1, pp. 18–24, 2006.

[36] G. Dorban, V. Defaweux, C. Demonceau, et al., "Interaction between dendritic cells and nerve fibres in lymphoid organs after oral scrapie exposure," *Virchows Archiv*, vol. 451, no. 6, pp. 1057–1065, 2007.

[37] F.-P. Huang, C. F. Farquhar, N. A. Mabbott, M. E. Bruce, and G. G. MacPherson, "Migrating intestinal dendritic cells transport PrPSc from the gut," *Journal of General Virology*, vol. 83, no. 1, pp. 267–271, 2002.

[38] M. Jeffrey, L. González, A. Chong, et al., "Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunochemical similarities can be resolved by immunohistochemistry," *Journal of Comparative Pathology*, vol. 134, no. 1, pp. 17–29, 2006.

[39] M. Jeffrey, L. González, A. Espenes, et al., "Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep," *Journal of Pathology*, vol. 209, no. 1, pp. 4–14, 2006.

[40] M. J. Ford, L. J. Burton, R. J. Morris, and S. M. Hall, "Selective expression of prion protein in peripheral tissues of the adult mouse," *Neuroscience*, vol. 113, no. 1, pp. 177–192, 2002.

[41] A. Aguzzi, F. L. Heppner, M. Heikenwalder, et al., "Immune system and peripheral nerves in propagation of prions to CNS," *British Medical Bulletin*, vol. 66, pp. 141–159, 2003.

[42] T. Blattler, S. Brandner, A. J. Raeber, et al., "PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain," *Nature*, vol. 389, no. 6646, pp. 69–73, 1997.

[43] M. Glatzel, F. L. Heppner, K. M. Albers, and A. Aguzzi, "Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion," *Neuron*, vol. 31, no. 1, pp. 25–34, 2001.

[44] S. L. Carlson, K. M. Albers, D. J. Belting, M. Parish, J. M. Conner, and B. M. Davis, "NGF modulates sympathetic innervation of lymphoid tissues," *Journal of Neuroscience*, vol. 15, no. 9, pp. 5892–5899, 1995.

[45] W. J. Hadlow, R. C. Kennedy, R. E. Race, and C. M. Eklund, "Virologic and neurohistologic findings in dairy goats affected with natural scrapie," *Veterinary Pathology*, vol. 17, pp. 187–199, 1980.

[46] W. J. Hadlow, R. C. Kennedy, and R. E. Race, "Natural infection of Suffolk sheep with scrapie virus," *Journal of Infectious Diseases*, vol. 146, pp. 657–664, 1982.

[47] M. H. Groschup, F. Weiland, O. C. Straub, and E. Pfaff, "Detection of scrapie agent in the peripheral nervous system of a diseased sheep," *Neurobiology of Disease*, vol. 3, no. 3, pp. 191–195, 1996.

[48] M. H. Groschup, M. Beekes, P. A. McBride, M. Hardt, J. A. Hainfellner, and H. Budka, "Deposition of disease-associated prion protein involves the peripheral nervous system in experimental scrapie," *Acta Neuropathologica*, vol. 98, no. 5, pp. 453–457, 1999.

[49] O. Andréoletti, P. Berthon, D. Marc, et al., "Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie," *Journal of General Virology*, vol. 81, no. 12, pp. 3115–3126, 2000.

[50] L. J. M. van Keulen, B. E. C. Schreuder, M. E. W. Vromans, J. P. M. Langeveld, and M. A. Smits, "Scrapie-associated prion protein in the gastrointestinal tract of sheep with natural scrapie," *Journal of Comparative Pathology*, vol. 121, no. 1, pp. 55–63, 1999.

[51] L. J. van Keulen, B. E. Schreuder, M. E. Vromans, J. P. Langeveld, and M. A. Smits, "Pathogenesis of natural scrapie in sheep," *Archives of Virology Supplementum*, no. 16, pp. 57–71, 2000.

[52] M. Jeffrey, S. Martin, L. González, et al., "Cell-associated variants of disease-specific prion protein immunolabelling are found in different sources of sheep transmissible spongiform encephalopathy," *Journal of General Virology*, vol. 84, pp. 1033–1045, 2003.

[53] L. J. M. van Keulen, M. E. W. Vromans, and F. G. van Zijderveld, "Early and late pathogenesis of natural scrapie infection in sheep," *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, vol. 110, no. 1, pp. 23–32, 2002.

[54] M. Jeffrey, S. Martin, J. R. Thomson, W. S. Dingwall, I. Begara-McGorum, and L. González, "Onset and distribution of tissue PrP accumulation in scrapie-affected Suffolk sheep as demonstrated by sequential necropsies and tonsillar biopsies," *Journal of Comparative Pathology*, vol. 125, no. 1, pp. 48–57, 2001.

[55] M. Jeffrey, S. Ryder, S. Martin, et al., "Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE). 1. Onset and distribution of disease-specific PrP accumulation in brain and viscera," *Journal of Comparative Pathology*, vol. 124, no. 4, pp. 280–289, 2001.

[56] G. McGovern and M. Jeffrey, "Scrapie-specific pathology of sheep lymphoid tissues," *PLoS One*, vol. 2, no. 12, article e1304, 2007.

[57] R. Heggebø, C. McL. Press, G. Gunnes, et al., "Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent," *Journal of General Virology*, vol. 81, no. 9, pp. 2327–2337, 2000.

[58] C. Ersdal, M. J. Ulvund, S. L. Benestad, and M. A. Tranulis, "Accumulation of pathogenic prion protein (PrPSc) in nervous and lymphoid tissues of sheep with subclinical scrapie," *Vet Pathol*, vol. 40, no. 2, pp. 164–174, 2003.

[59] R. Heggebø, L. González, C. McL. Press, G. Gunnes, A. Espenes, and M. Jeffrey, "Disease-associated PrP in the enteric nervous system of scrapie-affected Suffolk sheep," *Journal of General Virology*, vol. 84, no. 5, pp. 1327–1338, 2003.

[60] L. J. M. van Keulen, M. E. W. Vromans, C. H. Dolstra, A. Bossers, and F. G. van Zijderveld, "Pathogenesis of bovine spongiform encephalopathy in sheep," *Archives of Virology*, vol. 153, no. 3, pp. 445–453, 2008.

[61] R. Heggebø, C. McL. Press, G. Gunnes, L. González, and M. Jeffrey, "Distribution and accumulation of PrP in gut-associated and peripheral lymphoid tissue of scrapie-affected Suffolk sheep," *Journal of General Virology*, vol. 83, no. 2, pp. 479–489, 2002.

[62] S. J. Bellworthy, S. A. C. Hawkins, R. B. Green, et al., "Tissue distribution of bovine spongiform encephalopathy infectivity in Romney sheep up to the onset of clinical disease after oral challenge," *Veterinary Record*, vol. 156, no. 7, pp. 197–202, 2005.

[63] L. González, S. Martin, F. E. Houston, et al., "Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep," *Journal of General Virology*, vol. 86, no. 3, pp. 827–838, 2005.

[64] F. Houston, W. Goldmann, A. Chong, et al., "Prion diseases: BSE in sheep bred for resistance to infection," *Nature*, vol. 423, no. 6939, p. 498, 2003.

[65] S. Martin, L. González, A. Chong, F. E. Houston, N. Hunter, and M. Jeffrey, "Immunohistochemical characteristics of disease-associated PrP are not altered by host genotype or route of inoculation following infection of sheep with bovine spongiform encephalopathy," *Journal of General Virology*, vol. 86, no. 3, pp. 839–848, 2005.

[66] L. A. Terry, S. Marsh, S. J. Ryder, S. A. C. Hawkins, G. A. H. Wells, and Y. I. Spencer, "Detection of disease-specific Prp in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy," *Veterinary Record*, vol. 152, no. 13, pp. 387–392, 2003.

[67] A. Buschmann and M. H. Groschup, "Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle," *Journal of Infectious Diseases*, vol. 192, no. 5, pp. 934–942, 2005.

[68] G. A. Wells, M. Dawson, S. A. Hawkins, et al., "Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy," *Veterinary Record*, vol. 135, no. 2, pp. 40–41, 1994.

[69] G. A. H. Wells, S. A. C. Hawkins, R. B. Green, et al., "Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update," *Veterinary Record*, vol. 142, no. 5, pp. 103–106, 1998.

[70] G. A. H. Wells, J. Spiropoulos, S. A. C. Hawkins, and S. J. Ryder, "Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle," *Veterinary Record*, vol. 156, no. 13, pp. 401–407, 2005.

[71] C. Hoffmann, U. Ziegler, A. Buschmann, et al., "Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy," *Journal of General Virology*, vol. 88, no. 3, pp. 1048–1055, 2007.

[72] E. S. Williams, "Chronic wasting disease," *Veterinary Pathology*, vol. 42, no. 5, pp. 530–549, 2005.

[73] C. J. Sigurdson, "A prion disease of cervids: chronic wasting disease," *Veterinary Research*, vol. 39, no. 4, article 41, 2008.

[74] C. J. Sigurdson, T. R. Spraker, M. W. Miller, B. Oesch, and E. A. Hoover, "PrPCWD in the myenteric plexus, vagosympathetic trunk and endocrine glands of deer with chronic wasting disease," *Journal of General Virology*, vol. 82, no. 10, pp. 2327–2334, 2001.

[75] R. A. Bessen and R. F. Marsh, "Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters," *Journal of General Virology*, vol. 73, no. 2, pp. 329–334, 1992.

[76] J. C. Bartz, C. DeJoia, T. Tucker, A. E. Kincaid, and R. A. Bessen, "Extraneural prion neuroinvasion without lymphoreticular system infection," *Journal of Virology*, vol. 79, no. 18, pp. 11858–11863, 2005.

[77] A. F. Hill, R. J. Butterworth, S. Joiner, et al., "Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples," *The Lancet*, vol. 353, no. 9148, pp. 183–189, 1999.

[78] J. A. Hainfellner and H. Budka, "Disease associated prion protein may deposit in the peripheral nervous system in human transmissible spongiform encephalopathies," *Acta Neuropathologica*, vol. 98, no. 5, pp. 458–460, 1999.

[79] M. Glatzel, E. Abela, M. Maissen, and A. Aguzzi, "Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease," *New England Journal of Medicine*, vol. 349, no. 19, pp. 1812–1820, 2003.

[80] G. McGovern, S. Martin, L. González, J. Witz, and M. Jeffrey, "Frequency and distribution of nerves in scrapie-affected and unaffected peyer's patches and lymph nodes," *Veterinary Pathology*, vol. 46, no. 2, pp. 233–240, 2009.

[81] L. González, M. P. Dagleish, S. Martin, et al., "Ante-mortem diagnosis of preclinical sheep scrapie by immunohistochemical examination of rectal biopsy samples," *Veterinary Record*, vol. 162, no. 13, pp. 397–403, 2008.

[82] L. J. M. van Keulen, B. E. C. Schreuder, R. H. Meloen, G. Mooij-Harkes, M. E. W. Vromans, and J. P. M. Langeveld, "Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie," *Journal of Clinical Microbiology*, vol. 34, no. 5, pp. 1228–1231, 1996.

[83] M. Jeffrey, I. Begara-McGorum, S. Clark, et al., "Occurrence and distribution of infection-specific PrP in tissues of clinical scrapie cases and cull sheep from scrapie-affected farms in Shetland," *Journal of Comparative Pathology*, vol. 127, no. 4, pp. 264–273, 2002.

[84] L. González, S. Martin, S. Sisó, et al., "High prevalence of scrapie in a dairy goat herd: tissue distribution of disease-associated PrP and effect of PRNP genotype and age," *Veterinary Research*, vol. 40, no. 6, article 65, 2009.

[85] M. W. Head, D. Ritchie, N. Smith, et al., "Peripheral tissue involvement in sporadic, iatrogenic, and variant Creutzfeldt-Jakob disease: an immunohistochemical, quantitative, and biochemical study," *American Journal of Pathology*, vol. 164, no. 1, pp. 143–153, 2004.

[86] S. L. Benestad, J.-N. Arsac, W. Goldmann, and M. Nöremark, "Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology," *Veterinary Research*, vol. 39, no. 4, article 19, 2008.

[87] D. L. Felten, S. Y. Felten, and S. L. Carlson, "Noradrenergic and peptidergic innervation of lymphoid tissue," *Journal of Immunology*, vol. 135, supplement 2, pp. 755s–765s, 1985.

[88] D. L. Felten and S. Y. Felten, "Sympathetic noradrenergic innervation of immune organs," *Brain Behavior and Immunity*, vol. 2, no. 4, pp. 293–300, 1988.

[89] A. Bencsik, S. Lezmi, and T. Baron, "Autonomous nervous system innervation of lymphoid territories in spleen: a possible involvement of nonadrenergic neurons for prion neuroinvasion in natural scrapie," *Journal of Neurovirology*, vol. 7, no. 5, pp. 447–453, 2001.

[90] A. Bencsik, S. Lezmi, G. Hunsmann, and T. Baron, "Close vicinity of PrP expressing cells (FDC) with noradrenergic fibers in healthy sheep spleen," *Developmental Immunology*, vol. 8, no. 3-4, pp. 235–241, 2001.

[91] D. L. Felten, S. Y. Felten, D. L. Bellinger, et al., "Noradrenergic sympathetic neural interactions with the immune system: structure and function," *Immunological Reviews*, no. 100, pp. 225–260, 1987.

[92] S. Sisó, M. Jeffrey, and L. González, "Neuroinvasion in sheep transmissible spongiform encephalopathies: the role of the haematogenous route," *Neuropathology and Applied Neurobiology*, vol. 35, no. 3, pp. 232–246, 2009.

[93] M. W. Head, H. M. Yull, D. L. Ritchie, M. T. Bishop, and J. W. Ironside, "Pathological investigation of the first blood donor and recipient pair linked by transfusion-associated variant Creutzfeldt-Jakob disease transmission," *Neuropathology and Applied Neurobiology*, vol. 35, no. 4, pp. 433–436, 2009.

[94] G. C. Millson, R. H. Kimberlin, E. J. Manning, and S. C. Collis, "Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes," *Veterinary Microbiology*, vol. 4, no. 2, pp. 89–99, 1979.

[95] F. Houston, S. McCutcheon, W. Goldmann, et al., "Prion diseases are efficiently transmitted by blood transfusion in sheep," *Blood*, vol. 112, no. 12, pp. 4739–4745, 2008.

[96] S. Sisó, L. González, F. Houston, N. Hunter, S. Martin, and M. Jeffrey, "The neuropathologic phenotype of experimental ovine BSE is maintained after blood transfusion," *Blood*, vol. 108, no. 2, pp. 745–748, 2006.

[97] S. Sisó, L. González, M. Jeffrey, S. Martin, F. Chianini, and P. Steele, "Prion protein in kidneys of scrapie-infected sheep," *Veterinary Record*, vol. 159, no. 10, pp. 327–328, 2006.

[98] S. Sisó, M. Jeffrey, S. Martin, F. Houston, N. Hunter, and L. González, "Pathogenetical significance of porencephalic lesions associated with intracerebral inoculation of sheep with the bovine spongiform encephalopathy (BSE) agent," *Neuropathology and Applied Neurobiology*, vol. 35, no. 3, pp. 247–258, 2009.

[99] F. Houston, J. D. Foster, A. Chong, N. Hunter, and C. J. Bostock, "Transmission of BSE by blood transfusion in sheep," *The Lancet*, vol. 356, no. 9234, pp. 999–1000, 2000.

[100] N. Hunter, J. Foster, A. Chong, et al., "Transmission of prion diseases by blood transfusion," *Journal of General Virology*, vol. 83, no. 11, pp. 2897–2905, 2002.

[101] S. Sisó, M. Jeffrey, F. Houston, N. Hunter, S. Martin, and L. González, "Pathological phenotype of sheep scrapie after blood transfusion," *Journal of Comparative Pathology*, vol. 142, no. 1, pp. 27–35, 2010.

[102] C. K. Mathiason, J. G. Powers, S. J. Dahmes, et al., "Infectious prions in the saliva and blood of deer with chronic wasting disease," *Science*, vol. 314, no. 5796, pp. 133–136, 2006.

[103] C. A. Llewelyn, P. E. Hewitt, R. S. G. Knight, et al., "Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion," *The Lancet*, vol. 363, no. 9407, pp. 417–421, 2004.

[104] A. H. Peden, M. W. Head, D. L. Ritchie, P. J. E. Bell, and P. J. W. Ironside, "Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient," *The Lancet*, vol. 364, no. 9433, pp. 527–529, 2004.

[105] S. J. Wroe, S. Pal, D. Siddique, et al., "Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report," *The Lancet*, vol. 368, no. 9552, pp. 2061–2067, 2006.

[106] HPA Press Statement, "4th case of variant CJD infection associated with blood transfusion," Health Protection Agency, 2007, http://www.hpa.org.uk/hpa/news/articles/press_releases/2007/070118_vCJD.htm.

[107] HPA Press Statement, "vCJD abnormal prion protein found in a patient with haemophilia at post mortem," Health Protection Agency, 2009, http://www.hpa.org.uk/webw/HPAweb&HPAwebStandard/HPAweb_C/1234859690542?p=1231252394302.

[108] P. Saá, J. Castilla, and C. Soto, "Presymptomatic detection of prions in blood," *Science*, vol. 313, no. 5783, pp. 92–94, 2006.

[109] O. Andréoletti, C. Lacroux, A. Chabert, et al., "PrPSc accumulation in placentas of ewes exposed to natural scrapie: influence of foetal PrP genotype and effect on ewe-to-lamb transmission," *Journal of General Virology*, vol. 83, no. 10, pp. 2607–2616, 2002.

[110] C. Ligios, C. J. Sigurdson, C. Santucciu, et al., "PrPSc in mammary glands of sheep affected by scrapie and mastitis," *Nature Medicine*, vol. 11, no. 11, pp. 1137–1138, 2005.

[111] M. Vascellari, R. Nonno, F. Mutinelli, et al., "PrPSc in salivary glands of scrapie-affected sheep," *Journal of Virology*, vol. 81, no. 9, pp. 4872–4876, 2007.

[112] S. Sisó, M. Jeffrey, P. Steele, et al., "Occurrence and cellular localization of PrPd in kidneys of scrapie-affected sheep in the absence of inflammation," *Journal of Pathology*, vol. 215, no. 2, pp. 126–134, 2008.

[113] C. Lacroux, S. Simon, S. L. Benestad, et al., "Prions in milk from ewes incubating natural scrapie," *PLoS Pathogens*, vol. 4, no. 12, article e1000238, 2008.

[114] T. Konold, S. J. O. Moore, S. J. Bellworthy, and H. A. Simmons, "Evidence of scrapie transmission via milk," *BMC Veterinary Research*, vol. 4, article 14, 2008.

[115] L. González, S. Martin, S. A. Hawkins, et al., "Pathogenesis of natural goat scrapie: modulation by host PRNP genotype and effect of co-existent conditions," *Veterinary Research*, vol. 41, no. 4, p. 48, 2010.

[116] L. González, S. Martin, I. Begara-McGorum, et al., "Effects of agent strain and host genotype on PrP accumulation in the brain of sheep naturally and experimentally affected with scrapie," *Journal of Comparative Pathology*, vol. 126, no. 1, pp. 17–29, 2002.

[117] T. R. Spraker, R. R. Zink, B. A. Cummings, C. J. Sigurdson, M. W. Miller, and K. I. O'Rourke, "Distribution of protease-resistant prion protein and spongiform encephalopathy in free-ranging mule deer (Odocoileus hemionus) with chronic wasting disease," *Veterinary Pathology*, vol. 39, no. 5, pp. 546–556, 2002.

Hindawi Publishing Corporation
Interdisciplinary Perspectives on Infectious Diseases
Volume 2010, Article ID 876450, 4 pages
doi:10.1155/2010/876450

*Research Article*

# Proof That Chronic Lyme Disease Exists

**Daniel J. Cameron**

*Department of Medicine, Northern Westchester Hospital, Mt. Kisco, NY 10549, USA*

Correspondence should be addressed to Daniel J. Cameron, cameron@lymeproject.com

Received 11 December 2009; Accepted 26 March 2010

Academic Editor: Guey Chuen Perng



The evidence continues to mount that Chronic Lyme Disease (CLD) exists and must be addressed by the medical community if solutions are to be found. Four National Institutes of Health (NIH) trials validated the existence and severity of CLD. Despite the evidence, there are physicians who continue to deny the existence and severity of CLD, which can hinder efforts to find a solution. Recognizing CLD could facilitate efforts to avoid diagnostic delays of two years and durations of illness of 4.7 to 9 years described in the NIH trials. The risk to society of emerging antibiotic-resistant organisms should be weighed against the societal risks associated with failing to treat an emerging population saddled with CLD. The mixed long-term outcome in children could also be examined. Once we accept the evidence that CLD exists, the medical community should be able to find solutions. Medical professionals should be encouraged to examine whether: (1) innovative treatments for early LD might prevent CLD, (2) early diagnosis of CLD might result in better treatment outcomes, and (3) more effective treatment regimens can be developed for CLD patients who have had prolonged illness and an associated poor quality of life.

The evidence continues to mount that Chronic Lyme Disease (CLD) exists and must be addressed by the medical community if solutions are to be found. Thirty-four percent of a population-based, retrospective cohort study in Massachusetts were found to have arthritis or recurrent arthralgias, neurocognitive impairment, and neuropathy or myelopathy, a mean of 6 years after treatment for Lyme disease (LD) [1]. Sixty-two percent of a cohort of 215 consecutively treated LD patients in Westchester County were found to have arthralgias, arthritis, and cardiac or neurologic involvement with or without fatigue a mean of 3.2 years after treatment [2]. Klempner trials' subjects presenting with "well-documented, previously treated Lyme disease…had persistent musculoskeletal pain, neurocognitive symptoms, or dysesthesia, often associated with fatigue" and were ill during a mean of 4.7 years after onset [3]. Fallon trial subjects presenting with "well-documented Lyme disease, with at least 3 weeks of prior IV antibiotics, current positive IgG Western blot, and objective memory impairment," were ill during a mean of 9 years after onset [4]. Krupp LD subjects presented with "persistent severe fatigue at least 6 or more months after antibiotic therapy" [5].

There is also evidence that symptoms of CLD can be severe [4–8]. The Klempner trials described the quality of life for patients with posttreatment chronic Lyme disease (PTLD) as being equivalent to that of patients with congestive heart failure or osteoarthritis, and their physical impairment was "more than 0.5 SD greater than the impairment observed in patients with type 2 diabetes or a recent myocardial infarction" [3]. Fallon et al. described pain reported by patients with Lyme encephalopathy as being "…similar to those of postsurgery patients", and their fatigue "was similar to that of patients with multiple sclerosis." Limitations in physical functioning on a quality of life scale were "comparable with those of patients with congestive heart failure" [4].

Despite the above documented evidence, the 2006 Infectious Diseases Society of America (IDSA) LD treatment guideline panel questioned the existence of CLD [9]. The IDSA panel concluded, "Considerable confusion and controversy exist over the frequency and cause of this process and even over its existence" [9]. The IDSA panel referred to chronic manifestations of LD as Post-Lyme disease syndrome (PLDS), PTLD and CLD. There are shortcomings for each term. The PLDS nomenclature implies that an active LD has been successfully treated, that any remaining symptoms

are merely harmless vestiges of previous illness, and that the patient has been cured. The term PTLD merely implies that LD has been treated with antibiotics for 10 to 30 days. The CLD nomenclature implies that chronic manifestations of LD are present with or without evidence of active infection that cannot be reasonably explained by another illness.

There is no objective way to rule out an active infection. Lab tests that can be very helpful in confirming a clinical diagnosis of Lyme disease (such as the ELISA and Western blot tests) are not useful in determining whether the infection has been adequately treated. Common LD symptoms such as Bell's palsy, erythema migrans rash, meningitis, arthritis, or heart block, which are included in the current surveillance definitions, can be useful in "ruling in" Lyme disease, but the absence or disappearance of these symptoms cannot "rule out" an ongoing infection. A population-based, retrospective cohort study of individuals with a history of LD revealed that they were significantly more likely to have joint pain, memory impairment, and poor functional status due to pain than persons without a history of LD, even though there were no signs of objective findings on physical examination or neurocognitive testing [10]. Two recent mouse studies revealed that spirochetes persist despite antibiotic therapy and that standard diagnostic tests are not able to detect their presence [11, 12]. In sum, there are no clinical or laboratory markers that identify the eradication of the pathogen.

The IDSA panel also questioned the severity of CLD symptoms. The panel dismissed LD symptoms that persisted or recurred after their recommended, short-term course of treatment, stating that they were: "more related to the aches and pains of daily living rather than to either Lyme disease or a tickborne coinfection" [13]. The panel came to this conclusion despite four NIH retreatment trials that validated the severity of symptoms on 22 standardized measures of fatigue, pain, role function, psychopathology, cognition, and quality of life (QOL) [9].

Denying the existence and severity of CLD will continue to hinder the efforts to find a solution. Even in a prospective trial of LD, 10 to 16% of patients treated at the time of an erythema migrans rash remained symptomatic a mean of 30 months after treatment; the results varied depending on the duration of antibiotics treatment [14]. The actual failure rate in this prospective at 30 months is uncertain, given that 38% of the subjects were not evaluable due to poor adherence, receipt of intercurrent antibiotics, or development of a second episode of erythema migrans [14]. Patients infected with many other kinds of common bacteria—such as those that cause tuberculosis, bronchitis, or UTIs—can experience relapses after an initial course of antibiotic treatment fails or proves inadequate. Doctors routinely retreat patients who relapse in order to achieve a cure and prevent chronic symptoms. Why should patients with Lyme disease be treated differently?

The treatment failure rates could be exacerbated by diagnostic delays. Feder described treatment delays of six weeks in LD patients initially misdiagnosed with cellulitis [15]. In his trial, Fallon noted treatment delays averaging 2 years [4] without examining the causes of the delay. In my own practice, 32% of a consecutive case series of LD cases (confirmed by an ELISA and 5 or more positive bands on a IgG Western blot) had an average treatment delay of 1.8 years. [16] Of these, 60% conformed to Centers for Disease Control and Prevention (CDC) epidemiological criteria, presenting with a rash, Bell's palsy, or arthritis, yet, still had a diagnostic delay [16]. Patients in this case series were significantly more likely to fail their initial antibiotic treatment if they had delayed treatment [16]. Vrethem et al. concluded that patients treated because of neurological symptoms of LD were much more likely to present with persistent neuropsychiatric symptoms (headache, attention problems, memory difficulties, and depression) three years after treatment than a control group with erythema migrans (50% versus 16%, $P < .0001$) [17].

The diagnostic delays could reflect the failure to consider CLD in the differential diagnosis of chronic manifestations of LD. Steere did not include CLD in the differential diagnosis of patients seen in his university-based clinic. Instead, Steere diagnosed three-quarters of patients with "fibromyalgia" or "chronic fatigue syndrome" [18]. Similarly, Reid et al. did not include CLD as a diagnosis in their university LD clinic. Instead, he diagnosed these patients with "arthralgia-myalgia syndrome," primary depression, asymptomatic deer tick bites, osteoarthritis, and bursitis [16]. Hassett et al. diagnosed PTLD in patients with a history of objective evidence of LD, but withheld it from patients who lacked such a history. Instead, this group was diagnosed with "Chronic Multisymptom Illness (MUI) [19]. Their case definition for Chronic Multisymptom Illness was: " [having] at least one or more chronic symptoms from at least 2 of 3 categories of symptoms including musculoskeletal, fatigue, and mood cognition" that includes fibromyalgia, chronic fatigue syndrome, and Gulf War syndrome [19].

The risks to the individual and society of CLD have not been adequately considered [20]. As a group, CLD subjects in the four NIH trials had a 4% risk of a serious adverse event in the antibiotic treatment arms [4–6]. Yet, this risk has not been weighed against the risk CLD patients face if burdened with a long-term debilitating illness. The risk to society of emerging resistant organisms also has not been weighed against the societal risks associated with an emerging population saddled with CLD [8].

The economic burden of CLD has yet to be addressed. The mean cost estimate of CLD per patient in the US, of $16,199 per annum in 2002 dollars [8], reflects the toll on human health and cost to society. The annual per-patient cost of CLD is substantially higher than the cost for other common chronic illnesses: $10,911 for fibromyalgia [21], $ 10,716 for rheumatoid arthritis [21], and $13,094 for lupus [22]. Eighty-eight percent of the cost ($14,327) of Lyme disease consisted of indirect medical cost, nonmedical cost, and productivity losses. Cutting medical cost would save, at most, only 12% or $1,872 per annum. In 2002, the annual economic cost of LD in the US, based on the 23,000 cases reported to the CDC that year, was estimated to be $203 million [8]. Considering that the actual number of LD cases is believed to be 10 times higher than the number of cases reported to the CDC, the actual annual cost could be $2 billion [23, 24].

The burden of CLD is also reflected in testimony given by Connecticut's chief epidemiologist before the state's Public Health Department in 2004: "…roughly one percent of the entire population or probably 34,000 people are getting a diagnosis of Lyme Disease in Connecticut each year…20 to 25 percent of all families [in Connecticut] have had at least one person diagnosed with Lyme Disease ever and…three to five percent of all families have had someone diagnosed with Lyme Disease in the past year" [24].

No additional antibiotic trials have been planned for CLD patients despite the limitations of the Klempner and Fallon trials. Klempners' trials were limited by: (1) uncertainty over whether the initial antibiotic treatment was effective, (2) ongoing illness despite a mean of three previous treatments, (3) long onsets of illness averaging 4.7 years, (4) the poor quality of life of the subjects, and (5) small, underpowered sample sizes of 51 and 78 subjects [25]. The Fallon trial had similar limitations including: (1) uncertainty over whether the initial antibiotic treatment was effective, (2) treatment delays averaging two years, (3) onsets of illness averaging 9 years, (4) the severe pain, fatigue, psychopathology, and poor QOL of subjects, and (5) a small underpowered sample size of 37 subjects. The IDSA panel did not suggest any further clinical trials to address these limitations. In an editorial titled "Enough is Enough", which was published as a commentary on Fallon's trial, Halperin, an IDSA panel member, actually advised against further trials [26].

There is also an urgent need to address the mixed long-term outcome in children. Eleven percent of children with facial nerve palsy had persistent facial nerve palsy causing dysfunctional and cosmetic problems at 6-month followup [27]. Fourteen percent of 86 children had neurocognitive symptoms associated with or after classic manifestations of Lyme disease on followup [28]. Five of these children developed "behavioral changes, forgetfulness, declining school performance, headache or fatigue and in two cases a partial complex seizure disorder" [28]. Children with prior cranial nerve palsy have significantly more behavioral changes (16% vs. 2%), arthralgias and myalgias (21% vs. 5%), and memory problems (8% vs. 1%) an average of 4 years after treatment compared to controls [29].

Once we accept the evidence that CLD exists, the medical community should be able to find solutions. Professionals should be encouraged to examine whether: (1) innovative treatments for early LD might prevent CLD, (2) early diagnosis of CLD might result in better treatment outcomes, and (3) more effective regimens can be developed for CLD patients who have had prolonged illness and an associated poor quality of life.

## References


[1] N. A. Shadick, C. B. Phillips, E. L. Logigian, et al., "The long-term clinical outcomes of Lyme disease. A population-based retrospective cohort study," *Annals of Internal Medicine*, vol. 121, no. 8, pp. 560–567, 1994.

[2] E. S. Asch, D. I. Bujak, M. Weiss, M. G. E. Peterson, and A. Weinstein, "Lyme disease: an infectious and postinfectious syndrome," *Journal of Rheumatology*, vol. 21, no. 3, pp. 454–461, 1994.

[3] M. S. Klempner, L. T. Hu, J. Evans, et al., "Two controlled trials of antibiotic treatment in patients with persistent symptoms and a history of Lyme disease," *New England Journal of Medicine*, vol. 345, no. 2, pp. 85–92, 2001.

[4] B. A. Fallon, J. G. Keilp, K. M. Corbera, et al., "A randomized, placebo-controlled trial of repeated IV antibiotic therapy for Lyme encephalopathy," *Neurology*, vol. 70, no. 13, pp. 992–1003, 2008.

[5] L. B. Krupp, L. G. Hyman, R. Grimson, et al., "Study and treatment of post Lyme disease (STOP-LD): a randomized double masked clinical trial," *Neurology*, vol. 60, no. 12, pp. 1923–1930, 2003.

[6] M. S. Klempner, "Controlled trials of antibiotic treatment in patients with post-treatment chronic Lyme disease," *Vector Borne and Zoonotic Diseases*, vol. 2, no. 4, pp. 255–263, 2002.

[7] D. J. Cameron, "Clinical trials validate the severity of persistent Lyme disease symptoms," *Medical Hypotheses*, vol. 72, no. 2, pp. 153–156, 2009.

[8] X. Zhang, M. I. Meltzer, C. A. Pena, A. B. Hopkins, L. Wroth, and A. D. Fix, "Economic impact of Lyme disease," *Emerging Infectious Diseases*, vol. 12, no. 4, pp. 653–660, 2006.

[9] G. P. Wormser, R. J. Dattwyler, E. D. Shapiro, et al., "The clinical assessments treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America," *Clinical Infectious Diseases*, vol. 43, no. 9, pp. 1089–1134, 2006.

[10] N. A. Shadick, C. B. Phillips, O. Sangha, et al., "Musculoskeletal and neurologic outcomes in patients with previously treated Lyme disease," *Annals of Internal Medicine*, vol. 131, no. 12, pp. 919–926, 1999.

[11] E. Hodzic, S. Feng, K. Holden, K. J. Freet, and S. W. Barthold, "Persistence of Borrelia burgdorferi following antibiotic treatment in mice," *Antimicrobial Agents and Chemotherapy*, vol. 52, no. 5, pp. 1728–1736, 2008.

[12] H. Yrjänäinen, J. Hytönen, K.-O. Söderström, J. Oksi, K. Hartiala, and M. K. Viljanen, "Persistent joint swelling and borrelia-specific antibodies in Borrelia garinii-infected mice after eradication of vegetative spirochetes with antibiotic treatment," *Microbes and Infection*, vol. 8, no. 8, pp. 2044–2051, 2006.

[13] J. J. Halperin, E. D. Shapiro, E. Logigian, et al., "Practice parameter: treatment of nervous system Lyme disease (an evidence-based review): report of the quality standards subcommittee of the American Academy of Neurology," *Neurology*, vol. 69, no. 1, pp. 91–102, 2007.

[14] G. P. Wormser, R. Ramanathan, J. Nowakowski, et al., "Duration of antibiotic therapy for early Lyme disease: a randomized, double-blind, placebo-controlled trial," *Annals of Internal Medicine*, vol. 138, no. 9, pp. 697–704, 2003.

[15] H. M. Feder Jr. and D. L. Whitaker, "Misdiagnosis of erythema migrans," *American Journal of Medicine*, vol. 99, no. 4, pp. 412–419, 1995.

[16] M. C. Reid, R. T. Schoen, J. Evans, J. C. Rosenberg, and R. I. Horwitz, "The consequences of overdiagnosis and overtreatment of Lyme disease: an observational study," *Annals of Internal Medicine*, vol. 128, no. 5, pp. 354–362, 1998.

[17] M. Vrethem, L. Hellblom, M. Widlund, et al., "Chronic symptoms are common in patients with neuroborreliosis—a questionnaire follow-up study," *Acta Neurologica Scandinavica*, vol. 106, no. 4, pp. 205–208, 2002.

[18] A. C. Steere, E. Taylor, G. L. McHugh, and E. L. Logigian, "The overdiagnosis of Lyme disease," *Journal of the American Medical Association*, vol. 269, no. 14, pp. 1812–1816, 1993.

[19] A. L. Hassett, D. C. Radvanski, S. Buyske, S. V. Savage, and L. H. Sigal, "Psychiatric comorbidity and other psychological factors in patients with "chronic Lyme disease"," *American Journal of Medicine*, vol. 122, no. 9, pp. 843–850, 2009.

[20] D. J. Cameron, "Insufficient evidence to deny antibiotic treatment to chronic Lyme disease patients," *Medical Hypotheses*, vol. 72, no. 6, pp. 688–691, 2009.

[21] S. Silverman, E. M. Dukes, S. S. Johnston, N. A. Brandenburg, A. Sadosky, and D. M. Huse, "The economic burden of fibromyalgia: comparative analysis with rheumatoid arthritis," *Current Medical Research and Opinion*, vol. 25, no. 4, pp. 829–840, 2009.

[22] A. E. Clarke, J. M. Esdaile, D. A. Bloch, D. Lacaille, D. S. Danoff, and J. F. Fries, "A Canadian study of the total medical costs for patients with systemic lupus erythematosus and the predictors of costs," *Arthritis and Rheumatism*, vol. 36, no. 11, pp. 1548–1559, 1993.

[23] G. D. Ebel, E. N. Campbell, H. K. Goethert, A. Spielman, and S. R. Telford III, "Enzootic transmission of deer tick virus in new England and Wisconsin sites," *American Journal of Tropical Medicine and Hygiene*, vol. 63, no. 1-2, pp. 36–42, 2000.

[24] http://www.ct.gov/ag/lib/ag/health/0129lyme.pdf, p. 290,2008.

[25] D. J. Cameron, "Generalizability in two clinical trials of Lyme disease," *Epidemiologic Perspectives and Innovations*, vol. 3, article 12, 2006.

[26] J. J. Halperin, "Prolonged Lyme disease treatment: enough is enough," *Neurology*, vol. 70, no. 13, pp. 986–987, 2008.

[27] B. H. Skogman, S. Croner, M. Nordwall, M. Eknefelt, J. Ernerudh, and P. Forsberg, "Lyme neuroborreliosis in children: a prospective study of clinical features, prognosis, and outcome," *Pediatric Infectious Disease Journal*, vol. 27, no. 12, pp. 1089–1094, 2008.

[28] B. J. Bloom, P. M. Wyckoff, H. C. Meissner, and A. C. Steere, "Neurocognitive abnormalities in children after classic manifestations of Lyme disease," *Pediatric Infectious Disease Journal*, vol. 17, no. 3, pp. 189–196, 1998.

[29] M. Vázquez, S. S. Sparrow, and E. D. Shapiro, "Long-term neuropsychologic and health outcomes of children with facial nerve palsy attributable to Lyme disease," *Pediatrics*, vol. 112, no. 2, pp. e93–e97, 2003.