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Pathogenesis of neurotropic murine coronavirus is multifactorial

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Although coronavirus tropism is most often ascribed to receptor availability, increasing evidence suggests that for the neurotropic strains of the murine coronavirus mouse hepatitis virus (MHV), spike–receptor interactions cannot fully explain neurovirulence. The canonical MHV receptor CEACAM1a and its spike-binding site have been extensively characterized. However, CEACAM1a is poorly expressed in neurons, and the extremely neurotropic MHV strain JHM.SD infects *ceacam1a*$^{-/-}$ mice and spreads among *ceacam1a*$^{-/-}$ neurons. Two proposed alternative MHV receptors, CEACAM2 and PSG16, also fail to account for neuronal spread of JHM.SD in the absence of CEACAM1a. It has been reported that JHM.SD has an unusually labile spike protein, enabling it to perform receptor-independent spread (RIS), but it is not clear if the ability to perform RIS is fully responsible for the extremely neurovirulent phenotype. We propose that the extreme neurovirulence of JHM.SD is multifactorial and might include as yet unidentified neuron-specific spread mechanisms.

Introduction

Interruption of virus entry by targeting virus–receptor interactions has long been a goal of vaccination and antibody therapy, and more recently of small-molecule pharmaceutical therapy as well; there are now antiretroviral drugs targeting both the HIV envelope protein and its co-receptor CCR5. The species specificity of coronaviruses is most often attributed to receptor availability, so the cross-species transmission of severe acute respiratory syndrome (SARS) human coronavirus in 2002 focused a great deal of attention on the spike–receptor interaction as a target for therapeutic intervention [1]. However, our experience with neurotropic strains of the murine coronavirus mouse hepatitis virus (MHV, a model widely used for encephalitis and demyelinating disease; Box 1) suggests a paradox: although the spike protein is the most important determinant of neurovirulence [10,11], coronavirus neurotropism cannot be fully explained by receptor use. Two recent studies have confirmed this view. Mice lacking the canonical MHV receptor, CEACAM1a, remain susceptible to viruses expressing the spike protein from the extremely neurotopic JHM.SD (MHV-4) strain of MHV [12]. (JHM.SD is the most neurovirulent isolate [13] of the neurotropic JHM strain, also called MHV-4, which was derived by serial passage through mouse brain [14]). Although JHM.SD spreads efficiently among adjacent *ceacam1a*$^{-/-}$ neurons, no known alternative receptor is both expressed in neurons and capable of conferring MHV susceptibility to nonpermissive cells [15]. Here we review the known MHV receptor(s) and their spike protein-binding site, as well as the phenomenon of receptor-independent spread (RIS) performed by JHM.SD. Based on current knowledge, we believe that neither receptor use nor RIS can fully explain JHM.SD pathogenesis and hypothesize that the extreme neurotropism displayed by this strain must be multifactorial and include as yet unidentified neuron-specific spread mechanisms. Further studies of neurotropic MHV strains in *ceacam1a*$^{-/-}$ mice should clarify the mechanism(s) of MHV neurovirulence and guide future attempts to target the spike proteins of encephalitis viruses for therapeutic intervention.

The MHV spike glycoprotein binds the canonical receptor CEACAM1a

The canonical receptor for the murine coronavirus MHV, CEACAM1a, was one of the earliest virus receptors identified. It had long been noted that the SJL/J strain of inbred mice were resistant to MHV, whereas other strains (such as BALB/c) were susceptible. It was demonstrated that MHV binds to a 100–110-kDa protein in BALB/c tissues but not to SJL/J tissue extracts [16]. Inoculation of mice with a partially purified protein extract produced a monoclonal antibody, CC1 [17], which blocked MHV infection of cultured cells [18] and mice [19]. This antibody was used to further purify the receptor [17], and sequencing confirmed that it is a mouse carcinoembryonic antigen (CEA) family member [20] identical to the open reading frame of a transcript, mmCGM1, that was identified by screening a mouse cDNA library with a probe homologous to human CEA [21]. The same screen produced a second transcript, mmCGM2 [22], which was initially misidentified as a splice variant of mmCGM1 [23] and later identified as a different allele for which resistant SJL/J mice are homozygous [24]. (The cDNA library was derived from mice from the outbred CD-1 strain [21] that were apparently heterozygous at the receptor locus.) Although the SJL/J allele acts as an MHV receptor if over-expressed in tissue culture, it fails to bind MHV virions in virus overlay protein blot assays, and the soluble form has fourfold less virus neutralizing activity than the functional allele [25]. This suggests that it is too weak a receptor to function at endogenous levels; in addition, it is not recognized by CC1 [24]. The nomenclature was further complicated by the existence of multiple splice forms [26]; thus, in 1999, the nomenclature of the entire CEA family was revised, with the functional MHV receptor allele designated

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Box 1. Mouse hepatitis virus structural proteins

Coronaviruses are enveloped positive-sense RNA viruses that cause a variety of diseases in humans and animals, most notoriously the outbreak of severe acute respiratory syndrome (SARS) in 2002–2003. Mouse hepatitis virus is a coronavirus used as a model for both liver and CNS disease, facilitating studies of the viral pathogenesis of these organ systems in the natural host. The viral RNA genome is expressed as a set of seven nested mRNAs with a total of 11 open reading frames (ORFs) that encode two large replicase polyproteins (ORF1a and the frameshift product ORF1ab), three nonstructural proteins of unknown function (ORF2a, ORF4 and ORF5a) and six structural proteins: hemagglutinin esterase (HE; ORF 2b), spike (S; ORF3), envelope (E; ORF5b), membrane (M; ORF6), nucleocapsid (N; ORF7) and internal protein (I; alternative reading frame of ORF7) (Figure Ia). The HE and I proteins are not expressed by all strains of MHV. The structural proteins assemble at the ER–Golgi intermediate compartment (ERGIC), from which they are transported in vesicles to the plasma membrane to be released by exocytosis. The virus particles consist of a positive-sense RNA genome coated with N protein surrounded by an ERGIC-derived lipid bilayer envelope. The five remaining structural proteins are transmembrane proteins embedded in the viral envelope (Figure Ib). M, E, and I have small extracellular domains; HE forms dimers that project from the envelope as small spikes, and the larger, heavily glycosylated S protein forms trimers that project as large spikes or 'peplomers' that give coronaviruses their characteristic crown-like appearance by transmission electron microscopy. S protein mediates both attachment to the virus receptor and viral fusion with the cell membrane [2]. S is synthesized as a precursor that is cleaved post-translationally by cellular proteases into N-terminal S1 and C-terminal S2 subunits that remain noncovalently associated [3,4]. (The MHV-2 spike protein, which is not cleaved by the producing cell [5,6], is beyond the scope of this article.) The receptor-binding domain of S is associated with S1 and the fusion activity with S2 [7,8]. Although no definitive structure exists, MHV S is believed to be a type I viral fusion protein [9] (like influenza HA or HIV Env), which means that fusion activation should result in presentation of the hydrophobic fusion peptide by a three-stranded coiled-coil motif. Therefore, the fusion-activated conformation of S2 can be detected by aggregation or liposome binding.

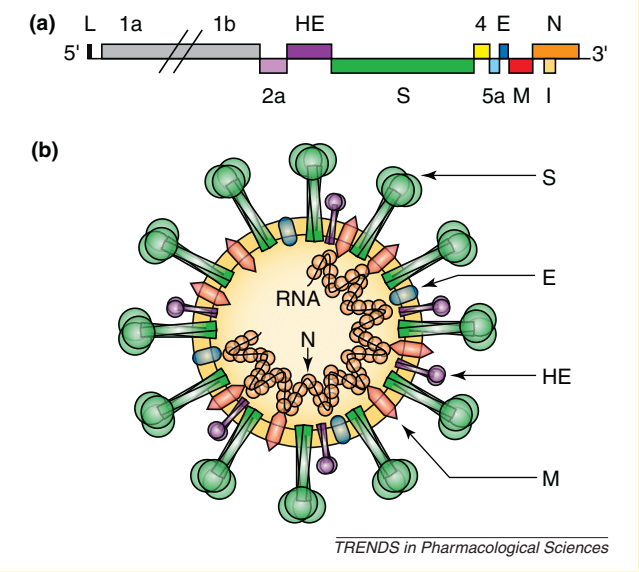

Figure I. MHV genome (a) and virus particle (b). Illustration created by Susan J. Bender and used with kind permission from Springer Science+Business Media: [58], Figure 1.

ceacam1a and the MHV-resistant SJL/J allele designated *ceacam1b* (Table 1) [27].

The MHV binding site on CEACAM1a has been extensively characterized. The murine CEA family belongs to the immunoglobulin superfamily and contains two branches: the transmembrane domain-anchored CEACAM proteins and the secreted pregnancy-specific glycoprotein (PSG) proteins. The extracellular portions of these proteins consist of different numbers of variable (V)- (usually N-terminal and designated N) and constant-type (C) immunoglobulin-like domains (divided into A and B subsets and numbered by subset), and many proteins have multiple splice variants [27]. CEACAM1a consists of a V-type N domain followed by either three (A1, B, A2) or one (A2) C-type domains, a transmembrane domain, and a long or short cytoplasmic tail. All four possible splice variants are expressed, generating CEACAM1a-4L, CEACAM1a-4S, CEACAM1a-2L, and CEACAM1a-2S (Figure 1, Table 1) [27]. All four forms support MHV infection in cultured cells [24]. It has been shown that both MHV and CC1 bind to the N domain [31], albeit at slightly different (if overlapping) sites: MHV requires amino acids 34–52 of CEACAM1a, whereas CC1 requires amino acids 1–70 and specifically residues 26-32, 42, and 43 [32]. Independent work identified the contiguous six-amino-acid motif at position 38–43 as crucial for MHV binding [33], and it is noteworthy that CEACAM1b has no homology with CEACAM1a at this sequence (Figure 2). Although the N domain is necessary and sufficient for neutralization and receptor activity [34], a truncated soluble protein containing only the N and A1 domains neutralizes less efficiently than either the two- or four-domain form, which suggests that the fourth Ig-like domain improves MHV binding [25]. The crystal structure of the soluble two-domain protein shows no interaction between the N and A2 domains; however, the critical MHV-binding residues 38–43 are prominently displayed

Table 1. Current and previously published names for mouse hepatitis virus receptors

New name	Isoform	Old name(s)
Ceacam1a	4L	$MHVR(4d)_L$ [24]; BgpD [26]
	4S	mCEA [21]; MHVR [28]; $MHVR_1$ [28]; mmCGM1 [21]; mmCGM1a [23]; BgpA [26]
	2L	BgpG [26]
	2S	BgpC [26]; MHVR(2d) [24]
Ceacam1b	4L	BgpF [26]
	4S	BgpE [26]
	2L	BgpH [26]
	2S	mmCGM2 [22]; mmCGM1b [23]; BgpB [26]
Ceacam2	2S	Bgp2C [29]; Bgp2(2d) [29]
Psg16		bCEA [30]

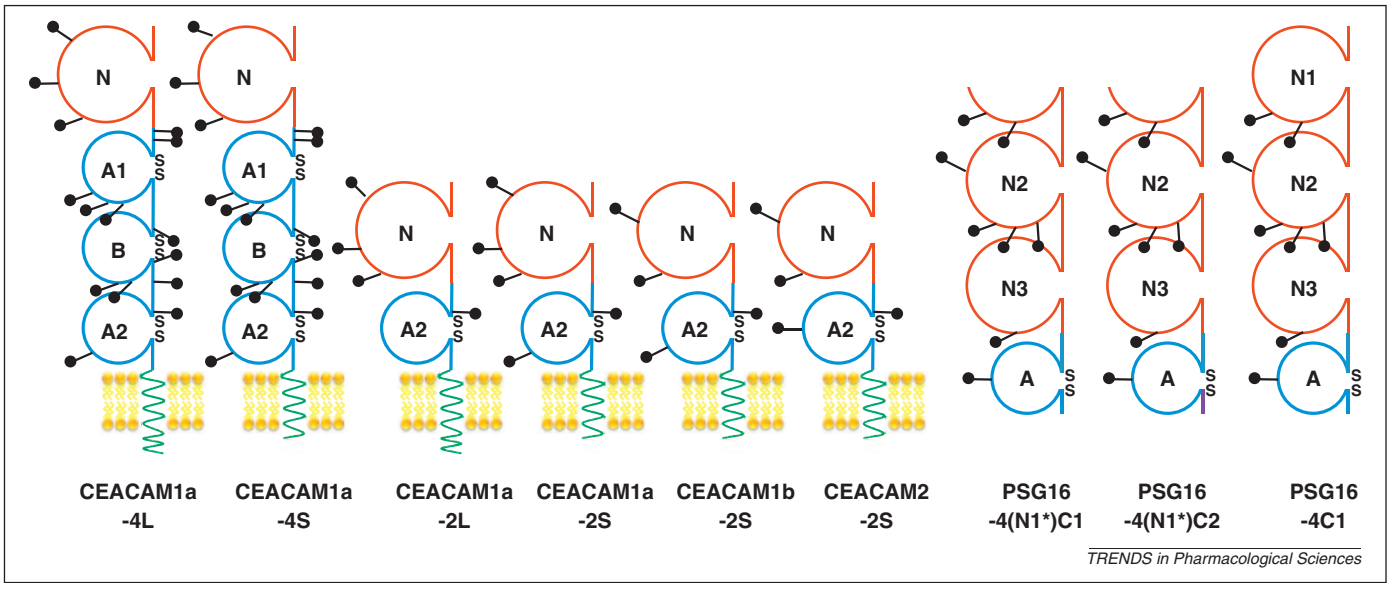

Figure 1. Comparison of domain structures of proposed mouse hepatitis virus receptors. V-type Ig-like domains are in red and C-type domains in blue. Transmembrane and cytoplasmic domains are in green and alternative C-terminus of PSG16-4(N1*)C2 in purple. Illustrations are modeled on those at the Carcinoembryonic antigen homepage (http://www.carcinoembryonic-antigen.de/index.html) with permission from W. Zimmermann.

on the CC′ loop of the N domain, which is stabilized in an unusually complex conformation relative to related proteins [35]. The ability of CEACAM1a to bind MHV is thus well understood.

No known MHV receptor explains the extreme neurotropism of MHV strain JHM

Although CEACAM1a is sufficient to confer MHV susceptibility to nonpermissive cell lines [28], CEACAM1a expression cannot account for some aspects of MHV tissue tropism. Notably, although some strains of MHV (including A59 and the highly neurotropic JHM) cause central nervous system (CNS) disease, CEACAM1a is poorly expressed in the CNS relative to other MHV target tissues such as the intestine and the liver [15,20]. Furthermore, neurons, which are the predominant CNS cell type infected by both A59 and JHM [11,15], express even lower levels of *ceacam1a* mRNA than other CNS cell types [15]. However, the ability of A59 to spread among wild-type but not *ceacam1a*$^{-/-}$ hippocampal neuron cultures implies that neurons do express CEACAM1a protein [15], although the possibility that A59 actually requires CEACAM1a expression by contaminating microglia cannot be ruled out. By contrast, the JHM.SD spike mediates spread even among *ceacam1a*$^{-/-}$ neurons, although initial infection rates are strikingly lower than for wild-type neurons [15]. At present, the role of CEACAM1a in MHV infection of the CNS is not clear.

One possible explanation for the discrepancy between CEACAM1a expression and MHV infection in neurons is that MHV uses an alternative receptor to infect neurons. This hypothesis is supported by the reduced affinity of the JHM spike for CEACAM1b relative to the A59 spike [25,36,37], which suggests that the receptor-binding

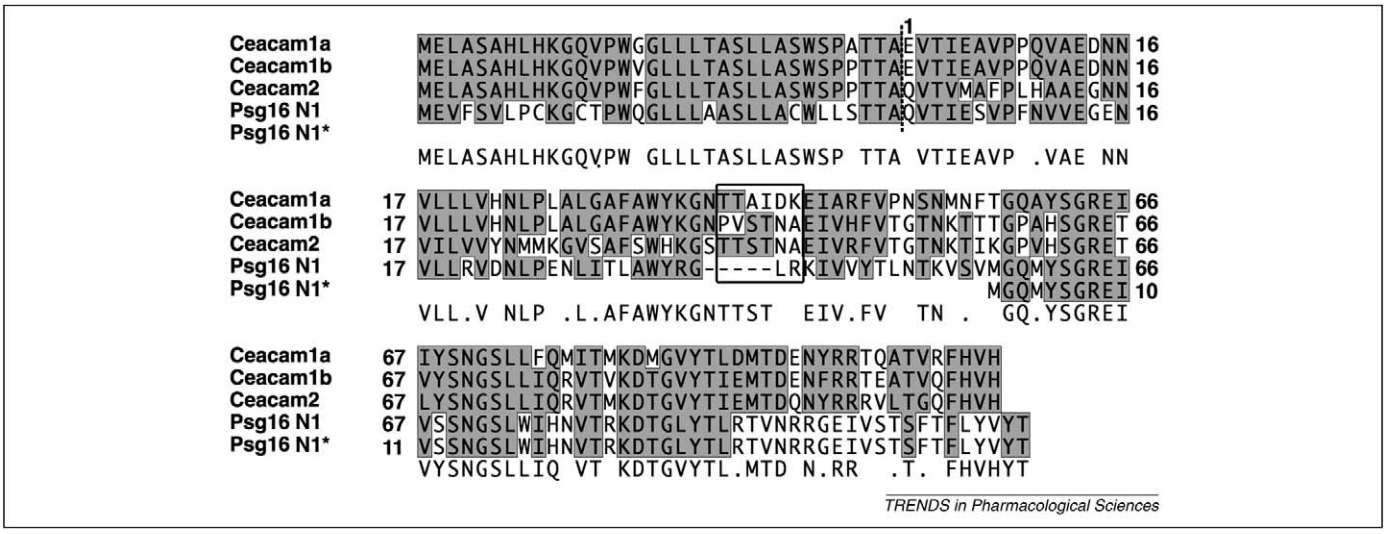

Figure 2. Alignment of the N-terminal domains of proposed mouse hepatitis virus receptors. Numbering is from the signal peptidase cleavage site (dotted line). The critical six-amino-acid motif (residues 38–43) required for MHV receptor activity is boxed.

domains of the two proteins differ in a biologically relevant fashion. Two CEA family members have been identified as possible alternative receptors: CEACAM2 and PSG16 (Figure 1). CEACAM2 (previously Bgp2) is similar to CEACAM1a in overall structure, but the MHV-binding loop at amino acids 38–43 has more homology to CEACAM1b (Figure 2). Like CEACAM1b, CEACAM2 can support MHV infection if it is overexpressed in nonpermissive cells [29], but the purified soluble form is less efficient at neutralizing MHV than is CEACAM1a [25]. Studies of the receptor efficiency of the two proteins have been hampered by an inability to control for receptor density, but it is generally believed that CEACAM2 is a less efficient receptor than CEACAM1a [15,29]. At present, it is not clear whether endogenous levels of CEACAM2 can support MHV infection. In addition, *ceacam2* mRNA is even more poorly expressed in neurons and glial cells than *ceacam1a* mRNA is [15]. Together, these data suggest that although CEACAM2 might be an alternative MHV receptor, it is not likely to account for the ability of JHM to spread in *ceacam1a*$^{-/-}$ neurons.

The other potential MHV receptor, PSG16, is both more promising and more problematic than CEACAM2. Unique among the PSG class, PSG16 is expressed in the CNS [15,30,38] and specifically in neurons [15], which makes it an attractive explanation for the ability of JHM to spread among *ceacam1a*$^{-/-}$ neurons. Two isoforms of PSG16 have been described (Figure 1) [30,38]. The first, PSG16-4(N1*)C1 (formerly known as bCEA; Table 1), was isolated from mouse brain by screening with a probe homologous to *ceacam1a* [30]. The second, PSG16-4(N1*)C2, was derived from sequencing of clones from a cDNA library derived from the mouse retina [38] and seems to result from joining of a cryptic splice donor site within the last exon of the –C1 isoform to an additional exon downstream. Both PSG16 isoforms lack the signal sequence and the N-terminal part of the N1 ectodomain relative to other murine PSG proteins (Figure 2). Both, like other PSG proteins, also lack any C-terminal membrane anchor motif. The absence of a membrane anchor does not necessarily preclude receptor activity: C-terminally truncated soluble forms of CEACAM1a were expressed on the cell surface, presumably by binding to membrane-anchored partners, and conferred MHV receptor activity to the expressing cells [34]. The N-terminal truncation is more problematic. Translocation in the absence of a signal sequence, although very uncommon, has been documented, but the PSG16 domain most homologous to the N domain of CEACAM1a is the N1 domain, which is missing the N-terminal MHV-binding site (Figure 2). An attempt to assess the MHV receptor activity of PSG16-4(N1*)C2 by targeting it to the cell surface with the signal sequence and membrane anchor domains of the avian retrovirus receptor TVA did not result in detectable surface expression, although parallel CEACAM1a and CEACAM2 constructs reached the cell surface and supported MHV infection [15]. Currently, it is not clear whether the chimeric PSG16 protein is specifically retained within the secretory pathway or is simply dysfunctional owing to misfolding. A third PSG16 isoform is predicted from analysis of the *Psg16* locus and placental expressed sequence tag libraries [39]. This full-length isoform, PSG16-4C1 (Figure 1), contains both a canonical signal sequence and an intact N1 domain and can be amplified from mouse placenta (J.M. Phillips, unpublished observations); however, alignment of the full-length PSG16 with murine CEACAM proteins shows that the MHV-binding motif on the CC′ loop has been entirely deleted (Figure 2). Additional studies are under way to determine whether this novel full-length isoform of PSG16 could be an alternative receptor for MHV.

The labile spike protein of the JHM strain can perform RIS

It is possible that the spike-dependent spread of JHM among *ceacam1a*$^{-/-}$ neurons relies not on an alternative receptor, but on a phenomenon known as RIS. The most common RIS assay involves overlaying a monolayer of nonpermissive cells with infected permissive cells; for RIS-competent strains of MHV (chiefly JHM.SD and the highly lethal JHM cl-2 isolate [40]), the infected cells fuse with neighboring uninfected cells, which fuse in turn to form large syncytia [41–43]. Neither the A59 strain [42] nor the mildly attenuated JHM.IA [44] performs RIS, and the acid-dependent JHM mutant OBLV60 performs RIS only if the medium is adjusted to the permissive pH [45]. RIS activity depends on the MHV spike glycoprotein but is not blocked by CC1 [45]. A similar assay has been used to study spread from infected microglia to neurons, although it is not clear that the latter truly lack CEACAM1a [15,46]. Fusion also occurs among nonpermissive cells expressing the spike protein alone and is blocked by anti-spike antibodies [45]. Taken together, these data imply that the RIS phenomenon requires only the JHM.SD spike protein.

It is thought that the ability of the JHM.SD spike to mediate RIS is due to the unusual instability displayed by this protein. Both A59 and JHM spike proteins are cleaved during egress into noncovalently associated S1 and S2 subunits (Box 1), and both proteins dissociate if the viruses are incubated at 37 °C under mildly alkaline pH, releasing soluble S1 and causing S2 to aggregate on the viral envelope [41,47]; a similar conformational change is observed in response to soluble CEACAM1a and correlates with increased liposome binding [37], which suggests that the conformational change observed at alkaline pH is the same as that responsible for receptor-dependent fusion. However, the JHM.SD spike dissociates more readily than that of A59 or JHM.IA [36,41,44], and mutations in the JHM.SD spike that increase stability decrease the ability to perform RIS [41,44]. In summary, the JHM.SD spike seems to have a more labile S1–S2 interaction than the A59 spike or RIS-incompetent JHM spikes [36], and this hyperlability correlates with RIS.

The extreme neurovirulence of the JHM.SD strain is multifactorial

Although neurons express very little MHV receptor and the highly neurotropic JHM.SD spike is capable of RIS, it is not fully clear that the ability to perform RIS is responsible for the extreme neurovirulence of JHM.SD. JHM.SD forms expanding foci of infected cells in hippocampal neuron cultures from wild-type or *ceacam1a*$^{-/-}$ mice, which suggests direct cell-to-cell spread, but these neurons do not

obviously form the syncytia [15] that are observed during RIS in cell lines. Several strains of JHM with mutations in S2 have lost the ability to perform RIS and are less neurovirulent than wild-type JHM.SD (J.C. Tsai, unpublished data) [48,49], but many are also deficient in CEACAM1a-dependent fusion [41,50,51], are less able to use CEACAM1b as an alternative receptor [52], or resist neutralization by soluble receptor (i.e. are not triggered by receptor binding) [53] despite wild-type receptor-binding domains, which implies defects in receptor-dependent fusion as well as RIS. The same is true of tissue-culture adapted strains that lack RIS activity due to large deletions in S1 [41]. By contrast, JHM.IA, which has four amino acid substitutions in its spike protein, including one (G310S) that abrogates RIS, has a stable spike and retains full CEACAM1a-dependent fusion activity. Although it is less pathogenic than JHM.SD, JHM1A is still relatively neurovirulent in naïve weanling and adult mice [44,54]. On the basis of these conflicting results, we suggest that the extreme neurovirulence of JHM.SD is multifactorial.

Although the extreme virulence of JHM.SD does not seem to depend on RIS alone, the ability to spread among neurons despite minimal CEACAM1a expression in that cell type could be crucial for MHV neurotropism. If so, infection of *ceacam1a*$^{-/-}$ mice and neurons will be an important tool for investigating the elements of neurovirulence. First, infection of *ceacam1a*$^{-/-}$ mice and neurons with JHM.IA and RIS-incompetent variants of JHM.SD should clarify whether CEACAM1a-independent spread in neurons is a special case of RIS or a new, neuron-specific phenomenon. If CEACAM1a-independent spread in neurons is distinct from RIS, the next question is whether JHM uses an alternative receptor for interneuronal spread or whether the synaptic environment enables JHM to spread in the absence of any receptor. The former might be identified by screening for neuronally expressed proteins that interact with JHM S or confer JHM infection to nonpermissive cells; the latter is more difficult to address, but determination of whether interneuronal spread requires cell-to-cell fusion or release of virus from neurites could suggest further approaches. These experiments should help to elucidate the mechanism of JHM.SD spread in *ceacam1a*$^{-/-}$ neurons.

Concluding remarks

An increasing body of research suggests that viruses that infect neurons might not use typical virus–receptor interactions for interneuronal spread; for example, both pseudorabies virus (which requires the attachment protein gD for extracellular but not interneuronal spread [55]) and measles virus (which uses a neurotransmitter receptor to spread *trans*-synaptically in the absence of its canonical receptor [56,57]) use alternative pathways for interneuronal spread. In these cases, drugs that target virus–receptor interactions might protect non-neuronal cells but might not prevent neuron-to-neuron spread of an established infection. The highly neurotropic strains of MHV offer a well-defined virus–receptor system for studying atypical interneuronal spread, and a better understanding of this system might suggest improved therapeutic targets for similarly atypical neuronotropic encephalitis viruses.

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