One proline deletion in the fusion peptide of neurotropic mouse hepatitis virus (MHV) restricts retrograde axonal transport and neurodegeneration

Saurav Saswat Rout¹, Manmeet Singh¹, Kenneth S. Shindler² and Jayasri Das Sarma¹

1. Department of Biological Sciences, Indian Institute of Science Education and Research, Kolkata, India
2. Scheie Eye Institute and FM Kirby Center for Molecular Ophthalmology, University of Pennsylvania, Philadelphia, PA, USA

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# Equal contribution

To whom correspondence should be addressed: Jayasri Das Sarma, Department of Biological Sciences, Indian Institute of Science Education & Research Kolkata, Mohanpur 741246, West Bengal, India. Email: dassarmaj@iiserkol.ac.in

Abstract

Mouse hepatitis virus (MHV; murine coronavirus [M-CoV]) causes meningoencephalitis, myelitis, and optic neuritis followed by axonal loss and demyelination. This murine virus is used as a common model to study both acute and chronic virus-induced demyelination in the central nervous system. Studies with recombinant MHV strains that differ in the gene encoding the spike protein have demonstrated that the spike has a role in both MHV pathogenesis and retrograde axonal transport. Fusion peptides (FPs) in the spike protein play a key role in MHV pathogenesis. In a previous study of the effect of deleting a single proline residue in the FP of a demyelinating MHV strain, we found that two central, consecutive prolines are important for cell–cell fusion and pathogenesis. The dihedral fluctuation of the FP was showed to be repressed whenever two consecutive prolines (PP) were present, in contrast to the presence of a single proline (P) in the chain. Using this proline-deleted MHV strain, here we investigated whether intracranial injection of this strain can induce optic neuritis by retrograde axonal transport from the brain to the retina through the optic nerve. We observed that the proline-deleted recombinant MHV strain is restricted to the optic nerve, is unable to translocate to the retina, and causes only minimal demyelination and no neuronal death. We conclude that an intact proline dyad in the FP of the recombinant demyelinating MHV strain plays a crucial role in translocation of the virus through axons and subsequent neurodegeneration

Introduction

MHV has been used extensively as a model to study acute and chronic virus-induced demyelination in the CNS for many years. Isogenic recombinant strains of MHV: RSA59 (demyelinating strain; DM) and RSMHV2 (non-demyelinating strain; NDM), which differ only in the spike gene generated by targeted RNA recombination between the parental DM strain MHV-A59 and parental NDM strain MHV-2, revealed that spike is important for pathogenesis (1). Intracranial inoculation of RSA59 and RSMHV2 have revealed their differential ability to induce macrophage infiltration, demyelination and axonal loss in spinal cord (2,3).

Interneuronal transport of virus and cell-cell fusion can be a critical check-point in mechanisms of demyelination and axonal loss in the spinal cord as well as in optic nerve. Spike protein plays a critical role in both virus-cell fusion, leading to infection, and pathological cell-cell fusion for spread, and spike can be a suitable target for antiviral therapy and virus-induced demyelination (2,4,5). Preventing spike protein interaction with the host cell may guide to design of therapeutic targets, but detailed study of the fusogenic determinant (minimal motif) of spike protein and its mechanistic
pathways are yet to be resolved. Previous study has shown the minimal motif, i.e. fusion peptide, plays an important role in cell-cell fusion (6-8), and includes two consecutive proline (PP) amino acids that putatively play a key role in the structure and function of the fusion peptide (8). The alteration of central proline amino acids within the putative fusion peptide has impaired the fusogenic property of RSA59, and subsequently alteration of fusogenicity has altered the demyelination pathology within the spinal cord during chronic stage of infection (8).

Site-directed mutagenesis of proline and generation of recombinant virus, revealed that one proline deletion, RSA59 (P) alters viral replication and also causes delayed syncytia formation from cell-to-cell fusion compared to RSA59 (PP) which contains the non-mutated parental strain MHV-A59 fusion protein (8). Altered fusogenicity consequently alters viral spread and pathogenicity. Acute stage pathology demonstrated that RSA59 (PP) was able to spread widely during acute encephalitis from brain to spinal cord, and cause demyelination; whereas, in the proline mutated strain RSA59 (P) viral antigen spread was mainly restricted to the inoculation site and meninges, and showed reduced demyelination (8).

MHV is reported to spread along neuronal axons in both anterograde and retrograde directions (3,9,10). Virus can reach and spread to proximal oligodendrocytes and myelin via anterograde axonal transport, resulting in demyelination in spinal cord white matter. Earlier studies suggested that demyelinating MHV strains reach the optic nerve via retrograde axonal transport (9,10), but whether the fusogenicity of virus mediates such transport is not well defined. In the optic nerve, the parental demyelinating strain MHV-A59 as well as RSA59 causes inflammation, demyelination, and axonal loss (ie, optic neuritis), in contrast to nondemyelinating parental MHV-2 and RSMHV2 strains (11,12), with clear evidence of retrograde viral spread in an MHV model of optic neuritis (ON) (11). The study demonstrated the ability of demyelinating MHV strains, and not non-demyelinating strains, to induce ON and axonal injury by retrograde axonal transport. Also, RSA59 contrasting to RSMHV2 can infect retinal ganglion cell (RGC) bodies in the retina following intracranial inoculation, and at the chronic stage, RSA59-infected mice show a significant loss of RGCs (13,14).

Whether retrograde transport of MHV to induce optic neuritis and damage RGCs is dependent on the capability of virus to cause cell-cell fusion is not known. Here, we compared the incidence and phenotype of optic neuritis after inoculation with fusogenic RSA59 (PP) and its fusion deficient proline mutated RSA59 (P) strain, and assessed retrograde axonal transport and RGC loss. RSA59 (PP) with direct capability of cell-cell fusion was able to reach optic nerve and RGCs, whereas fusion-deficient RSA59 (P) was not.

Results:

Both RSA59 (PP) and RSA59 (P) virus are able to replicate in optic nerve at day 3 and day 6 post-inoculation (p.i.)

Four-week-old male C57Bl/6 mice were intracranially inoculated with 50% LD50 doses of RSA59 (PP) and its mutated recombinant strain RSA59 (P). Optic nerves and whole eyes were harvested, fixed and sectioned. To detect the presence of viral antigens in the optic nerve, immunohistochemistry was performed with anti-Nucleocapsid (anti-N) antibody using avidin-biotin-peroxidase assay with substrate DAB. Images of immunostained optic nerves from mock-infected, RSA59 (PP)-infected and RSA59 (P)-infected mice were taken in 40X objective. At day 3 and 6 p.i. no viral staining was observed in mock-infected mice (Figure 1 a, d). At day 3 p.i. 100% of mice infected with RSA59 (PP) were positive for viral antigen staining in the optic nerve while ~75% of RSA59 (P) infected mice were positive for the same (Figure 1 b, c). No significant difference was found in the area of viral antigen staining between both strains (Figure 1 g) at day 3 p.i. At day 6 p.i., 100% infection was found in both infected strains (Figure 1 e, f); however, the area of viral antigen staining was higher in RSA59 (PP)- compared to RSA59 (P)-infected mice (Figure 1 h). At day 6 p.i., viral antigen in RSA59 (PP)-infected mice was spread throughout the optic nerve whereas in RSA59 (P)-infected mice the viral antigen spread was restricted to individual cells and meninges.

Distribution of microglia/macrophages in RSA59 (PP) and RSA59 (P) infected optic nerve

In order to determine whether the two strains could induce inflammation in the optic nerve, paraffin sections of the optic nerve were stained with H&E to evaluate the presence of
inflammatory cells as a sign of optic neuritis. Both RSA59 (PP) (75% of infected mice) and RSA59 (P) (50% of infected mice) virus strains induced optic neuritis (Figure 2 b, c), which was not present in mock-infected mice (Figure 2 a). In previous studies, most inflammatory cells in the optic nerve were found to be microglia/macrophages (11). In order to confirm similar findings here, optic nerve sections were immunostained with anti-Iba-1 antibody. In optic nerves from mice infected with either RSA59 (PP) or RSA59 (P) (Figure 2 e, f), a significant number of Iba1+ inflammatory cells (microglia/macrophage marker) were present compared to controls (Figure 2 d). There was no significant difference in the number and distribution of Iba1+ cells between mice infected with RSA59 (PP) and RSA59 (P) (Figure 2 g).

**RSA59 (PP) but not RSA59 (P) is able to infect retina**

Since viral antigen was detected in the optic nerve, we further investigated whether both viral strains have capability to traffic to retinal ganglion cell (RGCs) bodies from the site of inoculation (brain) in a retrograde manner. Whole eyes from mock-, RSA59 (PP)-, RSA59 (P)-infected mice were isolated and serially sectioned and immunostained with anti-N antibody and counterstained with hematoxylin. Images were captured in 40X magnification to look for viral antigen positive staining in the RGC layer. Interestingly, viral antigens were detected in ~75% of RSA59 (PP)-infected mouse eyes at day 3 p.i. and in all RSA59 (PP)-infected mouse retinas at day 6 p.i. (Figure 3 b, e); whereas, no viral antigen was detected in RSA59 (P)-infected (Figure 3 c, f) mouse retina, similar to mock-infected (Figure 3 a, d), at both 3 and 6 days p.i.. At day 3 p.i., RSA59 (PP) viral antigen was predominantly found in the ganglion cell layer (GCL), while at day 6 p.i. it spread through all layers of the retina. A significant difference was found in the area of viral antigen staining between RSA59 (PP) and RSA59 (P) strains at day 3 p.i. and 6 p.i. (Figure 3 g, h).

**Microglia/macrophage numbers increase significantly in the RGC layer at day 6 p.i.**

Microglia constitute a prominent part of the residential glial population in the retina. During maturation, they acquire their topographical distribution between inner plexiform layer (IPL) and outer plexiform layer (OPL) in the retina (15). To examine potential topographical changes after infection, 20μm thick cryopreserved sections were cut and immunofluorescently labeled with anti-Iba-1. Retinas from mock-infected mice showed resting ramified microglia in both IPL and OPL with only minimal presence in the GCL (Figure 4 a). In eyes from RSA59 (PP) infected mice, the population of microglia /macrophages was found to migrate and increase specifically in the GCL (Figure 4 b). However, in RSA59 (P) infected mouse retina, the microglia/macrophages seen in the GCL were comparatively less than in RSA59 (PP) infected mice (Figure 4 c, d). Across all retinal layers, there was a trend toward fewer total numbers of microglia/macrophages in mice infected with RSA59 (P) when compared to RSA59 (PP) infected mice at day 6 p.i., although this difference was not statistically significant (Figure 4 e).

**RSA59 (PP) induces more optic nerve demyelination and inflammation than RSA59 (P) at the chronic stage of optic neuritis**

In previous studies, mice infected with RSA59 showed chronic stage demyelination in the optic nerve (11). In order to evaluate demyelination in the current experiment, optic nerve sections were stained with LFB. In the chronic stage (day 30 p.i.), no demyelination was observed in mock-infected mouse optic nerves (Figure 5 a). However, 62.5% of optic nerves showed prominent demyelination (Figure 5 d) when mice were infected with RSA59 (PP), whereas a little or no demyelination was observed in RSA59 (P) infected mouse optic nerves (Figure 5 g, j). To evaluate inflammation at the chronic stage, optic nerve sections were stained with H&E. RSA59 (PP) infected mouse optic nerves exhibited inflammation at day 30 p.i. (Figure 5 e) whereas little or no inflammation was found in mice infected with RSA59 (P) (Figure 5 h) or in mock-infected mice (Figure 5 b). We characterized the inflammatory cells by staining optic nerve sections with anti-Iba1. At day 30 p.i., resting ramified microglia were found in mock infected mouse optic nerves (Figure 5 c). However, phagocytotic microglia/macrophage accumulation was found in numerous RSA59 (PP) (Figure 5 f) infected mouse optic nerves, but in RSA59 (P) (Figure 5 i) infected mice it was only occasionally found. Quantification of
this staining showed there was a significant increase in the total number of microglia/macrophages in RSA59 (PP) compared to mock infected and RSA59 (P) infected optic nerves (Figure 5 k).

**RSA59 (PP) induces RGC loss whereas RSA59 (P) does not**

Demyelinating optic neuritis induced by RSA59 has previously been shown to induce neuronal damage with RGC loss (13). To examine whether the proline mutated strain RSA59 (P) can cause any RGC loss at day 30, retinas from mock-infected, RSA59 (PP)-infected and RSA59 (P) infected mice were isolated and labelled with anti-Brn3a (RGC marker), and RGCs were counted in 12 standardized fields. The mice infected with RSA59 (PP) had a significant decrease in the RGC number compared to mock-infected mice (Figure 6 a, b), whereas infection with RSA59 (P) lead to no significant difference in RGC numbers compared to mock-infected mice (Figure 6 c, d).

**Discussion**

A previous study demonstrated that deletion of one proline within the fusion peptide of spike in the fusogenic MHV strain RSA59 reduced its ability to cause cell-cell fusion in vitro (8). In addition, the proline mutated strain RSA59 (P) showed reduced acute stage dissemination of virus within the brain and spinal cord, and reduced chronic stage demyelination in the white matter of spinal cord (8). Results of the current study comparing optic nerve and RGCs reveal that the proline mutated strain RSA59 (P) leads to reduced optic nerve inflammation and demyelination, suggesting significant consequences of differential virus replication and distribution as compared with RSA59 (PP) infection. It is also evident from the current study that RSA59 (PP), which contain the wild-type fusion peptide, causes severe RGC loss similar to previous studies of RSA59 [8], whereas RSA59 (P) fails to induce RGC loss. Thus, current results demonstrate a key role of dual prolines within the fusion peptide of spike in mediating pathology in optic neuritis. This is likely due to affects of the fusion peptide on the ability of virus to be transported along neuronal axons. Indeed, the presence of viral antigen in eyes of RSA59 (PP) infected mice suggests that retrograde axonal transport of virus from the site of inoculation occurred. In contrast, the lack of viral antigen in the retina of RSA59 (P) infected mice suggests that a single proline mutant strain of RSA59 was not capable of traveling into the eye. Although viral antigen was found in the optic nerve of RSA59 (P) infected mice, the absence of viral antigen in the eye suggests the reduced ability of RSA59 (P) to cause cell-cell fusion further reduced the degree of retrograde cell transport.

Demyelinating strain RSA59 (PP) successfully induced optic nerve inflammation as well as demyelination (11) and RGC loss (13) in accordance with our previous studies. Optic nerve inflammation was also evident in RSA59 (P) infected optic nerves in our current study, but the degree of demyelination and RGC loss was not significant in RSA59 (P) infected mice when compared to mock-infected mice. In addition, inflammation in the ganglion cell layer of RSA59 (PP) infected mouse retinas at the acute stage was comparatively more than in RSA59 (P) infected mice. Thus, results further support a key role of the fusion peptide in viral-induced neuroinflammation and demyelination. This may be a secondary effect due to reduced axonal transport of virus, or may be due to other direct effects of the mutated fusion peptide that remain to be elucidated in future studies.

Together, results suggest the lack of cell-cell fusion in the case of RSA59 (P) could play a major role in RGC loss induced during optic neuritis. This is the first report in our knowledge showing that a mutation which is known to reduce the ability of RSA59 (P) to cause cell-cell fusion also reduces its trafficking and infection to the RGC layer. This loss of pathologic function is likely governed by loss of proline altering rigidity and stability to the spike protein structure during fusion as shown in a previous study (8). The fusion property of the virus is important for its pathogenicity. From our previous study, it is evident that deletion of one proline from the fusion peptide may destabilize the spike protein which may result in impaired fusogenicity (8). Also, this single proline mutation results in reduced replication and spread of RSA59 (P). The interaction of the virus with axonal transport machinery needs to be further investigated. It has been suggested to either hijack cargo proteins to move along the microtubules, or can be transported inside vesicles, or can directly interact with the microtubules. How the virus enters and exits the neurons is also not yet clear, but there is a possibility that the virus may gain access to the
nerve endings due to the traumatic disruption at the site of inoculation. The presence of viral antigen in the RGC layer found here is consistent with our prior study suggesting that the virus travels through retrograde axonal transport (11). Although infection in RGCs alone cannot exclude hematogenous spread as an alternative mechanism from retrograde transport, the distinct timing and amount of viral spread between the optic nerve (seen by day 3 p.i.) and the retina (seen by day 6 p.i.) suggests retrograde axonal transport to be the more likely mechanism of transport.

From the above proline insertion-deletion study, it is evident that proline, a single amino acid may serve as an important entity in the fusion peptide of MHV required for efficient retrograde axonal transport and demyelination. Dissecting the minimal essential motif of the spike protein fusion peptide may enable us to design a mimetic peptide to set a stage for competition to reduce virus induced neuroinflammation and RGC loss. Overall, data suggest that the presence of two consecutive prolines plays a major role in preventing spread of virus to post synaptic targets (RGCs).

**Materials and Methods**

**Mice** Four-week-old MHV free C57BL/6J male mice (In vivo Biosciences, Bangalore) were used for the experiment. All the procedures were carried out in accordance with ethical guidelines approved by the Institutional Animal Care and Use Committee at the Indian Institute of Science Education and Research Kolkata. The animal protocols adhered to the guidelines of the CPCSEA, India.

**Viruses** Proline mutated recombinant virus RSA59 (P) that has been engineered from RSA59 (PP), both expressing enhanced green fluorescent protein (EGFP) were used as described in previous studies (8). Mice were monitored up to day 30 p.i.

**Inoculation of mice** 20,000 pfu (50% LD50) of RSA59 (PP) and RSA59 (P) were used to inoculate 4-week-old, MHV free, C57BL/6J (2,8) mice. Desired viruses were diluted from the stock solution using 0.75% PBS/BSA and a final volume of 20μl of diluted virus was inoculated intracranially (IC). Virus is injected directly into the brain by syringe needle through the skull posterior to the orbits near lateral geniculate nuclei (LGN). Upon IC inoculation virus infects neurons in regions containing RGC axonal projections, including the LGN within the thalamus (Figure 7) (11). Animals were euthanised using isofluorane and perfused with 1X PBS followed by 4% paraformaldehyde (PFA) and tissues were harvested at day 3 and day 6 p.i. for acute stage analysis and at day 30 p.i. for chronic stage study. 4-6 mice were used per infection type per day post-infection of study, as indicated in each corresponding data figure. For mock infection, 3 mice per day post infection were used and inoculated with 0.75% PBS/BSA.

**Histopathology** At day 3, 6 and 30 p.i. optic nerves and eyes were isolated from mock infected as well as MHV infected mice. For paraffin sectioning, optic nerves and eyes were post-fixed with 4% PFA for 15 mins, processed and embedded with paraffin. For cryosectioning, eyes were post-fixed with 4% PFA for 15 mins, processed in 10% and 30% sucrose solution and embedded with OCT medium (Tissue Tek, Hatfield, PA), sectioned sagittally with the help of a cryotome (Thermo Scientific) to 20μm thickness, and mounted on charged glass slides. Right eye was collected for immunohistochemistry on paraffin sections with viral nucleocapsid antigen whereas the left eye was collected for immunofluorescence on cryosections with Iba1. 5μm thin optic nerve sections were cut and stained with H&E, and additional sections of day 30 p.i. optic nerves were stained with LFB to detect myelin damage.

**Immunohistochemical analysis** Serial cross sections of optic nerves and eyes were stained using the avidin-biotin-immunoperoxidase technique (Vector Laboratories, Burlingame, CA) with 3, 3’ diaminobenzidine as substrate and anti-Iba1 (Wako Chemicals, USA. Cat no. 019-19741) and anti-N (Nucleocapsid protein of MHV-JHM, monoclonal clone 1-16-1, kindly provided by Julian Leibowitz, Texas A&M, College Station, TX) antibody as primary antibodies.

**Immunofluorescence of frozen sections** Frozen tissue sections were washed with PBS at room temperature to remove cryomatrix. Tissues then were incubated for 1 h at room temperature with 1M glycine in PBS to reduce nonspecific cross-linking, followed by a 10-min incubation at room temperature with 1 mg/ml
NaBH4 in PBS to reduce autofluorescence. Slides were washed with PBS and incubated with blocking serum containing PBS with 0.5% Triton X-100 and 2.5% goat serum. The sections were incubated overnight at 4 °C with a primary anti-Iba1 antibody diluted in blocking serum, washed, and subsequently incubated with Alexa fluor 568-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific) diluted in PBS with goat serum for 2h at room temperature. All incubations were carried out in a humidified chamber. After PBS washing, sections were mounted with DAPI containing mounting medium (Vectashield) and were imaged using Nikon eclipse Ti2 microscope. The images were processed with Fiji (ImageJ 1.52g) software.

Quantification of histological slides

Quantitative analysis of anti-viral antigen staining was quantified as described previously using Fiji (ImageJ 1.52g) software (8,16). Briefly, 5 fields of each optic nerve tissue section were captured at 40X magnification and analyzed. 4-5 optic nerve sections per mouse per infection were used to measure the area of staining. Similarly, 4-5 sections of retina each spanning from medial to peripheral per mouse per infection were scanned at highest magnification of 40X. The RGB images were color-deconvoluted into three different colors to separate the DAB-specific staining. To ensure that all labeled cells were selected, a threshold value was defined for each image. The magnitude of staining was defined as the area of staining and plotted. Each dot in a graph represents the average value of the captured frames from each mouse. To ensure error-free data collection, the entire quantification procedure was performed by two investigators together and read in a blinded manner.

The magnitude of Iba1+ microgliosis/macrophage activation was defined as the number of Iba1+ cells present in total area. 4-5 number of optic nerve tissue sections per mouse per infection were randomly selected to count the total number of Iba1+ cells. Similarly, 4-5 number of slices of immunofluorescent labeled retina spanning from medial to peripheral per mouse per infection were selected to count the total number of Iba1+ cells and number of Iba1+ cells present in GCL layer.

To determine the total white matter area and areas with myelin loss in day 30 RSA59 (PP) and RSA59 (P) p.i. mice, 4-5 LFB-stained optic nerve sections from each mouse were randomly selected and analyzed using Fiji software (ImageJ 1.52g) (8,17). The total area of the demyelinating plaque was outlined and measured. The percentage of demyelination per section per mouse was obtained by dividing the total area of the demyelinating plaque over the total area of the optic nerve section and then multiplied by 100.

Quantification of RGC numbers

RGC immunolabeling and quantification was performed as described in our previous studies (13). Briefly, both eyes at day 30 p.i. were removed and fixed with 4% PFA. Retinas were then isolated and washed 3 times with 1X PBS, permeabilised with 0.5% triton X-100 at -70°C. Retinas were incubated in blocking buffer (PBS containing 2% goat serum and 2% Triton X 100) for 4 hr at room temperature. Then they were incubated overnight with anti Brn3a antibody (Synaptic systems, Germany. Cat no. 411 003) diluted 1:500 in blocking buffer at 4°C. After washing, retinas were incubated for 2 hr at room temperature with Texas red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200. Photographs of RGCs were taken in 12 standardized fields at 1/6, 3/6, and 5/6 of the retinal radius from the center of the retina in four quadrants at 40X magnification. RGCs were counted in each field by a blinded investigator using Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD) software.

Statistical analysis

The level of significance for immunohistochemistry staining and LFB staining in spinal cord sections was calculated using one-way ANOVA and Tukey’s multiple comparison test. All data were scatter plotted with standard deviation and analyzed using GraphPad prism 6.01 software. Each dot in a graph represents the average values acquired from each mouse. A p-value <0.05 was considered statistically significant and presented as *,p.

Data availability

All experimental procedures in detail are available in Material and Method section and all experimental data including the schematic presentation of the inoculation site are available in the Result section.
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Competing Financial Interests

The authors have no competing financial interests.

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Figures and Figure legends

**Figure 1.** Virus replication (viral antigen positive cells) in RSA59 (PP) and RSA59 (P) infected mouse optic nerve sections during the acute stage of infection (a-f). Longitudinal 5μm thick sections of mock, RSA59 (PP) and RSA59 (P) infected optic nerve were immunohistochemically stained with anti-N (viral antigen) antibody (N=4 mice/virus). The mock-infected optic nerve of both day 3 (a) and day 6 p.i. (d) showed negative staining to anti-N. Intracranially infected RSA59 (PP) optic nerve sections showed positive staining to anti-N at both days 3 (b) and 6 p.i. (e). Mice infected with RSA59 (P) also showed positive staining to anti-N at both day 3 (c) and day 6 p.i. (f). The amount of anti-N staining was quantified as stated in methods section. The staining was higher in RSA59 (PP) infected mouse optic nerve compared to mock-infected on day 3 (g) and day 6 (h) p.i.. A significant difference in staining was observed between RSA59 (PP) and RSA59 (P) at day 6 p.i.. Level of significance was determined by one-way ANOVA and Tukey’s multiple comparison test. *p<0.05; **p<0.01; ***p<0.001.
Figure 2. Differential optic nerve inflammation at day 6 (acute stage) p.i. by H&E staining (a, b, c) and Iba-1 staining (d, e, f). Optic nerve sections from mock-, RSA59 (PP)- and RSA59 (P)-infected mice (N=4 mice/virus) harvested day 6 p.i. were stained with H and E. Both RSA59 (PP) (b), and RSA59 (P) (c) infected optic nerves showed accumulation of inflammatory cells (arrows) compared with mock-infected mice (a). Similarly, sequential sections stained with anti-Iba-1 (microglia/macrophage marker) antibody showed a few resident Iba-1+ cells in optic nerves of mock-infected mice (d) whereas in both RSA59 (PP) (e) and RSA59 (P) infected mice (f) higher numbers of Iba1+ cells were found. Iba1+ cells were counted in 40X images. Significantly more Iba1+ cells were present post infection with RSA59 (PP) and RSA59 (P) compared to mock infection. No significant difference was observed in inflammation levels between RSA59 (PP) and RSA59 (P) infected mice (g). Level of significance was determined by one-way ANOVA and Tukey’s multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001
Figure 3. Differential virus antigen distribution in RSA59 (PP) and RSA59 (P) infected mouse retina sections during the acute stage of infection (a-f). Longitudinal 5μm thick whole eye sections of mock (a, d), RSA59 (PP) (b, e) and RSA59 (P) (c, f) infected mice were immunohistochemically stained with anti-N (viral antigen) antibody (N=4 mice/virus), and photographs of representative retinas are shown. Mock-infected mouse retina sections on both day 3 (a) and day 6 (d) p.i. showed no anti-N staining, whereas RSA59 (PP) infected mouse retina sections showed anti-N staining at both days 3 (b) and 6 (e) p.i.. RSA59 (P) infected mouse retina showed no anti-N staining at both day 3 (c) and day 6 p.i.(f). The amount of anti-N staining was quantified as stated in methods section. Significantly more staining was observed in RSA59 (PP) infected mouse retina compared to mock-infected and RSA59 (P) on day 3 (g) and day 6 p.i. (h). Level of significance was determined by one-way ANOVA and Tukey’s multiple comparison test. *p<0.05; **p<0.01; ***p<0.001. (GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer).
Figure 4. Iba1+ microglial cells migration through different layers of retina. Eye sections stained with Iba-1 antibody show resting microglia morphology in mock- (a), and similar morphology in RSA59 (P)-infected mice (c), while Iba-1+ cell morphology is more pronounced in RSA59 (PP) (b) infected retina. The few Iba1+ cells in mock-infected eyes were scattered across retinal layers, including the inner plexiform layer (IPL), and ganglion cell (GCL) (arrows), and in RSA59 (P)-infected mice (e) similarly few Iba-1+ cells were found in the GCL (arrows). A higher number of Iba-1+ cells were found in the GCL of RSA59 (PP) (b) infected retina (arrows). The total number of Iba1-1+ cells present in GCL of the retina per section was counted (d). The number of Iba1+ cells in the GCL was higher in RSA59 (PP) than mock-infected mice. In RSA59 (P) infected mice there was no significant increase in Iba-1+ cells. Similarly, total number of Iba1+ cells present in all the layers of retina were counted (e). Despite an appearance of more Iba1+ cells present post infection with RSA59 (PP) compared to mock infection in the representative sections, this trend was not significant, and no significant difference was observed between RSA59 (PP) and RSA59 (P) infected mice. Level of significance was determined by one-way ANOVA and Tukey’s multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. (N=3-6 mice/group). (GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer).
Figure 5. Differential demyelination and inflammation of RSA59 (PP) and RSA59 (P) infected mouse optic nerve sections during the chronic stage of infection (a-j). Longitudinal optic nerve sections from mock (a), RSA59 (PP) (d) and RSA59 (P) (g) infected mice harvested on day 30 p.i. stained with Luxol Fast Blue (LFB) showed demyelination in almost all RSA59 (PP) (d) infected optic nerves. Mice infected with RSA59 (P) (g) showed small patches of demyelinated areas. Similarly, sections stained with H and E to assess the level of inflammation showed a higher level of inflammation in RSA59 (PP) (e) and a lower level of inflammation in RSA59 (P) (h), which was comparable to mock-infected (b). Similarly, sections stained with anti-Iba-1 antibody showed an accumulation of Iba1+ cells in RSA59 (PP) (f) infected optic nerve but few Iba-1+ cells in mock (c) and RSA59 (P) (i) infected optic nerve. The percentage area of myelin (LFB stain) in the optic nerve was calculated by densitometry and showed greater myelin loss in RSA59 (PP) infected mice compared to the mock-infected optic nerve. RSA59 (P) infected mice had no significant myelin loss compared to mock infected controls (j). Total number of Iba1+ cells were counted and showed a significant difference between mock-infected control
and RSA59 (PP) as well as RSA59 (PP) versus RSA59 (P) (k). Level of significance was determined by one-way ANOVA and Tukey’s multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. (N=3-6 mice/group)

Figure 6. Comparative RGC loss in RSA59 (PP) and RSA59 (P) infected retina during the chronic stage of infection (a-c). Significant RGC loss was found in RSA59 (PP) (b) infected eyes at day 30 p.i. compared to mock-infected mice (a). Mice inoculated with RSA59 (P) (c) did not show any significant RGC loss compared to mock-infected control mice (a). RGCs were counted from 12 standardized retinal fields. The average number of RGCs surviving per eye shows RSA59 (PP) induced a significant decrease in RGC number compared to mock-infected mice, while RSA59 (P) did not cause any significant decrease in RGC number (d). Level of significance was determined by one-way ANOVA and Tukey’s multiple comparison test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. (N=5 mice/group)
Figure 7. Schematic diagram demonstrating the route of virus transport into the retina. Virus is injected directly into the brain by syringe/25G needle through the skull posterior to the orbits. Virus infects neurons in regions containing RGC axonal projections, including the lateral geniculate nucleus (LGN) within the thalamus. Viral antigen is then transported retrogradely along RGC axons to the RGC cell bodies in the retina. SC, superior colliculus.
One proline deletion in the fusion peptide of neurotropic mouse hepatitis virus (MHV) restricts retrograde axonal transport and neurodegeneration
Saurav Saswat Rout, Manmeet Singh, Kenneth S Shindler and Jayasri Das Sarma

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