Axon Myelin Transfer of a Non-Envelope Virus

Jean-Pierre Roussarie1*, Claude Ruffie1, Julia M. Edgar2, Ian Griffiths2, Michel Brahic1*

1 Department of Virology, URA3015 Centre National de la Recherche Scientifique (CNRS), Institut Pasteur, Paris, France, 2 Applied Neurobiology Group, Institute of Comparative Medicine, University of Glasgow, Glasgow, United Kingdom

We showed previously that Theiler’s virus, a neurotropic non-enveloped picornavirus of mouse, traffics from the axon of infected neurons into the surrounding myelin. When this traffic is interrupted, as in the shiverer mouse which bears a mutation in the myelin basic protein gene, the virus is unable to persist in the central nervous system. In the present work, we used the Wlds mutant mouse, a strain in which axonal degeneration is considerably slowed down, to show that axon to myelin traffic takes place in the absence of axon degeneration. Our results suggest the existence of a mechanism of transfer of axonal cytoplasm into the myelin which Theiler’s virus might exploit to ensure its persistence.

INTRODUCTION

Myelin is an extension of the oligodendrocyte cell body wrapped many times around the axon. Myelin is important not only for rapid saltatory conduction of nerve impulse but also for support of the underlying axon [1]. For example, inactivating the Cnp1 myelin gene does not alter myelin ultrastructure but causes accumulation of membranous organelles in the axons, axon swelling and degeneration [2]. This support role, and the exact way by which oligodendrocytes and axons communicate, is still poorly understood. Theiler’s virus (TMEV) is a mouse picornavirus responsible for a persistent infection of CNS and a chronic, inflammatory, neurological disease [3]. TMEV infects neurons first but persists in oligodendrocytes and macrophages in the white matter. In a previous paper we showed that TMEV infects retinal ganglion neurons when injected in the eye, is transported axonally in the optic nerve, and infects optic nerve oligodendrocytes through their myelin sheaths [4]. We also showed that a deletion of the myelin basic protein gene prevents this axon-myelin traffic and renders the mice resistant to persistent infection [4,5]. This observation raises important questions regarding the mechanism of axon to myelin traffic and the role of this traffic in viral persistence. Picornaviruses, such as ‘TMEV’, are non-enveloped and cannot travel from cell to cell by budding followed by fusion. Classically, they exit infected cells by lysis, although exit without lysis has been discussed but not formally proven [6]. In the present work we investigated the role of axon lysis in the entry of TMEV into the myelin using the Wlds mouse, a mutant strain in which axonal degeneration is 10 times slower than in wild type controls [7]. We show that TMEV enters the myelin even when the axons do not degenerate.

RESULTS

The Wlds mutation does not affect the infection of retina by TMEV or the axonal transport of the virus in the optic nerve

Wild type and Wlds mice were inoculated with TMEV in the vitreous chamber of the eye as described [4]. First, we examined if the Wlds mutation altered the infection of retinal ganglion neurons. The amount of viral negative-strand RNA in retina was measured by real-time RT-PCR 2 days post-inoculation. Negative-strand RNA is diagnostic of viral replication, as opposed to genomic positive-strand RNA which can come from the inoculum as well as from newly replicated genomes. No difference was observed between the 2 strains of mice (Figure 1). Next we examined the effect of the Wlds mutation on the axonal transport of the virus by looking for viral antigens in neurons of the lateral geniculate nucleus (LGN), to which retinal ganglion cells project, 4 days post-inoculation. Serial sections of the entire nucleus were stained with fluorescent antibodies specific for TMEV capsid antigens and the total number of positive cell bodies was counted. As shown in Figure 2, no statistically significant difference was found between wild type and Wlds mice. This result suggested that the mutation did not affect the rate of anterograde axonal transport of the virus.

We then studied the effect of infection on axonal degeneration using both immunofluorescence with the SMI32 monoclonal antibody, an antibody which stains non-phosphorylated neurofilaments specifically, and electron microscopy. Wild type and Wlds optic nerves were obtained 3 and 4 days post-inoculation, coded and observed blindly. In the wild type mice, both at 3 and 4 days post-inoculation, immunofluorescence showed conspicuous staining with the SMI32 antibody and electron microscopy showed numerous swollen axons with disorganized cytoskeleton. In contrast, in Wlds mice, no SMI32 staining was observed by immunofluorescence and abnormal axons were observed only very occasionally by electron microscopy (Figure 3A, B). Thus, the Wlds mutation did not affect the infection of the retina by TMEV or the axonal transport of the virus in the optic nerve but prevented axonal degeneration.

* To whom correspondence should be addressed. E-mail: mbrahic@stanford.edu

1 Current address: Molecular and Cellular Neuroscience, Rockefeller University, New York New York, United States of America
2 Current address: Department of Microbiology-Immunology, Stanford University School of Medicine, Stanford, California, United States of America
The axon to myelin traffic of TMEV takes place in the absence of axon degeneration

We then asked if oligondrocytes in the optic nerves of WldS mice were infected 4 days post-inoculation, the earliest time at which infected glial cells are detected in this nerve in wild type mice. An early time was chosen to rule out the possibility of gli to gli spread of the infection. The mice were inoculated in one eye only in order to use the contralateral pre-chiasmatic segment of the optic nerve as control for the hematogenous spread of virus from axon to myelin. Frozen sections were coded, doubly-stained with fluorescent anti-astrocyte, an astrocyte marker, and with fluorescent anti-TMEV antibodies. Optical sections obtained with the Apotome fluorescent microscope were scanned blindly, and doubly-labeled cells were quantified as described [4]. No virus was found in the contralateral pre-chiasmatic segment, ruling out hematogenous spread. In contrast, infected cells were present in the ipsilateral nerve in both wild type and WldS mice. The percentage of infected cells that were CNPase+ was the same for wild type and WldS mice (65%±8% and 75%±7% respectively; p=0.31, Mann-Whitney test) (Figure 3C). The CNPase+ infected cells were GFAP+ astrocytes. The number of infected oligodendrocytes per mm² of optic nerve section was also the same for both mice (1.1±0.3 and 1.3±0.2 oligodendrocytes/mm²; p=0.40, Mann-Whitney test) (Figure 3D). Therefore the WldS mutation did not prevent the infection of optic nerve oligodendrocytes from the axons of retinal ganglion neurons.

DISCUSSION

Our results show that the WldS mutation does not alter the extent of replication of TMEV in retina or the axonal transport of the virus from retinal ganglion cells to neurons of the LGN. They show a dramatic difference in axonal degeneration between the optic nerves of intra-ocularly inoculated wild type and WldS mice. In spite of this difference, Figure 3 shows that the extent of oligodendrocytes cell body was counted. Each circle corresponds to a different mouse. There is no statistically significant difference of viral load between control and WldS mice (p=0.40, Mann-Whitney test).

MATERIALS AND METHODS

Mice

C57BL/6OlaHsd-Wld, referred to in this paper as WldS mice, and their C57BL/6jOlaHsd controls were purchased from Harlan, UK. All mice used were 4 week-old females.
Experimental conditions to monitor axon-myelin traffic

Virus preparation, intraocular inoculation, quantification of viral load in the retina, preparation of optic nerve frozen sections, staining of the sections with fluorescent antibodies and microscopic analysis of the sections were described in detail previously [4].

Analysis of the infection in the LGN

To measure infection in the LGN, 4% paraformaldehyde (PFA) fixed brains obtained as described previously [4] were infiltrated with 30% sucrose. Serial sections of the entire thalamus were prepared using a Leica sliding microtome. Free floating sections were stained for viral antigens using a 1/1000 dilution of an anti-TMEV capsid hyperimmune rabbit

Figure 3. Phenotype of TMEV-infected optic nerves of wild type and Wld^s mice. A: Longitudinal sections of optic nerves reacted with the SMI32 antibody (green). Nuclei were stained with DAPI (blue). SMI32 staining was detected in wild type samples only. B: Electron microscopy. Swollen axons with disorganized cytoskeleton are present in wild type mice only (star); bar = 5 μm. Mice shown in A and B were sacrificed 3 days post inoculation. C: Frozen sections were stained for CNPase (oligodendrocytes) or GFAP (astrocytes) and for TMEV to determine the percentage of TMEV^+ cells which were CNPase^+. No difference between wild type and Wld^s mice was found (p = 0.31, Mann-Whitney test). D: The same sections were used to measure the density of CNPase^+, TMEV^+ cells (infected oligodendrocytes/mm^2). No difference between wild type and Wld^s mice was found (p = 0.40, Mann-Whitney test). In C and D, each circle corresponds to a different mouse.

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Detection of unphosphorylated neurofilaments with the SMI32 antibody

Anesthetized mice were perfused with PBS followed by buffered neutral formaldehyde (BNF) (33 mM NaH2PO4, 45 mM K2HPO4, 4% PFA). Mice were left intact for 1 hour. Optic nerves were then dissected out, post-fixed in BNF overnight at 4°C, and embedded in paraffin. Ten 10μm sections were cut with a microtome. Slides were baked at 56°C for 2 days, then deparaffinized and incubated for 40 minutes at 96°C in 10 mM sodium citrate buffer (pH6). They were then washed in water, blocked for 1h in PBS, 10% fetal calf serum (FCS) and 0.5% bovine serum albumin (BSA). The sections were incubated overnight at 4°C with the SMI32 antibody (Sternberger Monoclonals) diluted 1:200 in PBS, 10% FCS, 0.5% BSA. After washing, the sections were incubated for 1 hour with an Alexa 488 coupled anti-mouse antibody diluted 1:200 in PBS. Finally, the sections were mounted in Vectashield supplemented with DAPI (Vector). Sections were coded and observed blindly. All samples classified as showing axonal degeneration were from wild type mice upon decoding.

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Electron Microscopy

Samples were prepared for electron microscopy as described previously [13]. To assess axonal degeneration, EM grids of inoculated and control wild type and WldS nerves were coded. Ten randomly selected regions imaged at x2.5k to x6.7k initial magnification were examined for change. All injected wild type and WldS nerves were correctly identified as morphologically affected or normal, respectively.

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Author Contributions

Conceived and designed the experiments: MB JR. Performed the experiments: MB CR JR JE IG. Analyzed the data: MB JR JE IG. Wrote the paper: MB JR.