MHV-JHM INFECTIONS OF RODENT NEURONAL CELLS: REPLICATION AND TRAFFICKING OF STRUCTURAL PROTEINS AND PROGENY VIRIONS

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INTRODUCTION

A number of studies have signified the importance of neurons in the pathogenesis of the acute as well as chronic forms of MHV-induced neurologic disease in rodents. Following intranasal inoculation, MHV strains A59 and JHM invade the murine CNS by way of the olfactory and trigeminal nerves.[1,2,3] Accordingly, interneuronal spread was hypothesized to be the most likely mechanism to explain viral CNS penetration by this route. Subsequent spread to other CNS regions was shown to also involve specific neuronal populations and tracts.[2,4] Immunohistochemistry and in situ hybridization have demonstrated tropism of MHV-A59 for neurons within the olfactory nuclei, nuclei of the amygdala, central tegmental nucleus, entorhinal cortex, subiculum, claustrum, lateral habenular nucleus, subthalamic nucleus, basal ganglia, substantia nigra and septal nuclei among others in mice[2,5] and of JHMV for hippocampal and cerebellar Purkinje neurons in rats.[6,7] Although JHMV inoculated rats can develop a delayed onset, demyelinating encephalomyelitis with no prior evidence of acute encephalitis, histopathological and in situ hybridization studies have implied that clinically silent neuronal involvement likely precedes the development of the more chronic, demyelinating form of disease.[6,8] In addition to their potential importance as vehicles for MHV penetration and spread and as targets for cytopathic processes, neurons may also serve as reservoirs for viral persistence. The suggestion has been made that neurons may in fact be particularly well suited for this, due to insufficient expression of MHC class I molecules on their surfaces thus enabling them to avoid recognition by virus specific cytotoxic T cells.[9] For these reasons, studies focusing on the interactions between neurotropic strains of MHV and rodent neurons should enhance our understanding of neuropathogenic mechanisms in a fundamental way.

Assembly of JHMV and MHV-A59 virions in fibroblastic cells occurs within the

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perinuclear region by budding into a smooth membrane compartment transitional between the rough endoplasmic reticulum and Golgi apparatus.[10,11] Furthermore, budding from this site was concluded to be controlled both temporally and spatially by the accumulation of the viral M structural protein.[11] Similar results were found with infection of murine pituitary tumor AtT20 cells where progeny virions were observed to exit cells via the constitutive rather than the regulated exocytic pathway.[12] Progeny JHM virions have likewise been shown to mature in close association with the Golgi apparatus in cultured mouse spinal cord neurons.[13]

The mechanism by which progeny virions exit infected cells in these examples likely involves a microtubule-dependent process. In this regard neurons are strikingly specialized. Each neuron is characteristically asymmetric, possessing two different types of neurites each performing specific functions. The single axon is responsible for conducting electrical signals away from the cell soma, while several dendrites serve as the main signal reception apparatus. Associated with this polarity in neurite function, are underlying differences in their membrane proteins and cytoskeletal frameworks.[14] Furthermore, since the biosynthetic functions of the neuron are restricted to the somato-dendritic domain, movement of materials to and from the apical or axonal domain, which may extend more than a meter in length, must involve an efficient process. This is accomplished by two microtubule-dependent processes: fast transport of membranous organelles and slow transport of cytosolic and particularly cytoskeletal proteins.[14] The present study deals with the growth and trafficking of JHMV structural proteins and virions in two rodent neuronal systems: cultured rat hippocampal neurons and murine OBL21 neuronal cells.[15]

RESULTS AND DISCUSSION

Embryonic day 18 to 20 Wistar Furth rats were used for the preparation of dissociated hippocampal neuron cultures following previously described procedures[16] with minor modifications. Cells were plated on either poly-L-lysine coated glass coverslips or plastic petri dishes at densities ranging between 3×10^4 to 6×10^4 viable cells per cm^2 and grown in a serum-free medium (DMEM and Ham's F12 1:1 with N2 supplements[17] plus Na pyruvate). Type-1 astrocytes were grown in co-culture with hippocampal neurons to provide trophic support and to buffer against the excitotoxic effects of the amino acid neurotransmitter glutamate. The astrocyte feeders, which were physically separated from neuronal cultures, were grown as monolayers in 60 mm petri dishes for neurons situated on 12 mm diameter glass coverslips, or grown on 24.5 mm diameter Costar transwell inserts (catalogue # 3425) for neuronal cultures situated in Costar 6 well cluster plates.

Hippocampal neurons were inoculated with JHMV at between 9 and 11 days in vitro (D.I.V.) with moi's that ranged between 5 and 30 pfu/cell. At this stage in culture most axons and dendrites have matured in terms of their functional and molecular properties and synaptogenesis is well under way.[16] In addition to wt JHMV, two variants, ATllf cord and V5A13 (88), which possess overlapping deletions of 441 and 447 nucleotides respectively within the S1 portion of the S gene[18,19] were also used. These variants were of special interest to us due to their reported attenuation in neurovirulence as judged by the shift in the pattern of disease they induced from one of fatal encephalomyelitis to chronic demyelination.[20,21] Moreover, this shift was suggested to result from the loss of tropism for neurons but not glia.[20] This system consequently provided an ideal opportunity to assess their tropism directly.

Cell density appeared to be a more important determinant than moi with respect to establishing and maintaining viral growth in hippocampal neuronal cultures. In addition, although infection could be established with all three viruses, the number of neurons initially infected and subsequent growth tended to be greater with the two variants (Table 1).

Table 1. Growth of wt JHMV and S Deletion Variants in Dissociated Hippocampal Neuron Cultures.

		PFU/ml of Culture Supernatant* Days Post-Inoculation				
		1	2	3	4	5
Experiment # 1[a]	wt JHMV	1.6 ±0.5	4.4 ±2.0	12.6 ± 8.0	47.0 ±12.7	55.5 ±17.7
	AT11f cord	3.1 ±0.1	33.0 ±12.1	116.0 ± 26.5	485.0 ± 57.3	239.7 ± 37.9
Experiment # 2[b]	wt JHMV	0.2 ±0.2	<0.1	0	0.6 ±1.0	1.4 ±2.4
	AT11f cord	1.6 ±1.0	3.3 ±1.2	2.1 ±1.0	15.8 ± 9.7	42.3 ± 2.3
	V5A13 (88)	4.1 ±1.1	12.0 ± 6.2	16.6 ± 6.1	48.7 ±22.6	79.0 ± 9.0

*Expressed as x 10^2
[a]In Experiment # 1 cells dispersed from E20 Wistar Furth rat hippocampi were seeded at a density of 60,000 viable cells/cm^2. At 11 D.I.V. cultures were inoculated with virus at a moi of 5 pfu/cell.
[b]In Experiment # 2 cells dispersed from E20 Wistar Furth rat hippocampi were seeded at a density of 30,000 viable cells/cm^2. At 10 D.I.V. cultures were inoculated with virus at a moi of 30 pfu/cell.

Thus, if in vitro virus-neuron interactions accurately reflect the situation occurring in vivo, one can make the provisional conclusion that loss of neuronal tropism is not associated with these truncations involving the S glycoprotein. Furthermore, although our wt JHMV expresses the HE glycoprotein,[19] the variants do not,[19] suggesting that this glycoprotein is likely not necessary for neuronal infections. Hence, the neuroattenuation associated with these variants in vivo, may not be easily explained by a shift in tropism. In some culture preparations endogenous astrocytes proliferated to represent a significant proportion of the total cell population. However, they did not usually become infected to any significant extent until after 2 dpi, a finding in agreement with our previous work.[22] Furthermore, astroglial infections were usually associated with the formation of syncytia while neuronal infections were not.

Viral proteins appeared to localize to both tapering and branching dendritic-like as well as fine axonal-like processes of infected neurons. Dual immunolabelling with antibodies specific for either N or S and tau was carried out to further assess the distribution of viral proteins to the two neuronal domains (Fig.1).

Tau along with MAP2 are microtubule-associated proteins which appear to form cross-bridges between adjacent microtubules.[23] Immunocytochemical studies on cultured rodent neurons have demonstrated that tau and MAP2 topographically segregate to predominantly axonal and somato-dendritic domains respectively[24] in agreement with the situation existing in vivo.[25] This is slightly complicated by the fact that axons tend to arise most commonly as branches from the proximal portions of dendrites rather than directly from the cell soma in hippocampal neurons grown in culture,[26] and that during the early stages of culture, MAP2 and tau immunoreactivity co-distribute within

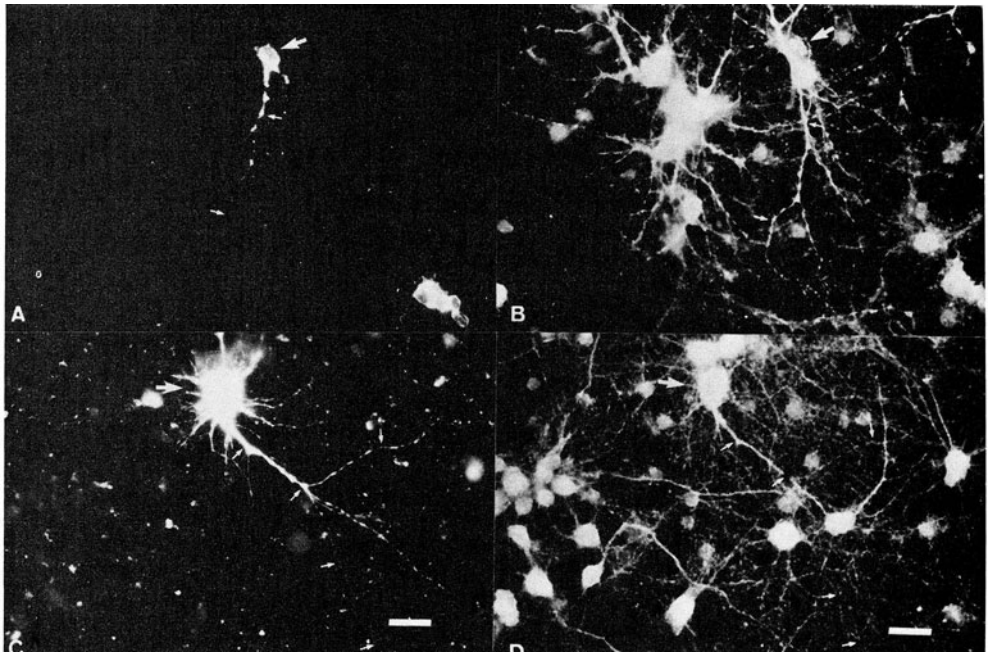

Figure 1. E20 hippocampal neuronal cultures infected with V5A13 (88) variant at 10 D.I.V. and immunocytochemically labelled ldpi. A, an infected neuron is labelled by using MAb 4B6.2 specific for N and FITC conjugated secondary antibody. B, cells in the same field as A are labelled with a rabbit polyclonal anti-tau antibody (Sigma) and Texas Red conjugated secondary antibody. C, an infected neuron is labelled with MAb 5B17.0 specific for S and FITC conjugated secondary antibody. D, identical field to that in C is labelled as described for B. Arrows point to complementary neurites in A and B and C and D. Bar represents 19 μm.

both axon- and dendrite-like neurites.[24,27] However, in cultures in which prominent fasciculation of axons occurred, viral N and S immunoreactivity could be localized within axon bundles, eliminating some of this ambiguity. Electron microscopic examination of cultures revealed the association of nucleocapsid core components with microtubule arrays as reported previously.[22] Occasionally virions were observed within neurites (Fig. 2). Studies of JHMV infected polarized ependymal cells indicate that progeny exit the cell baso-laterally judging by the pattern of spread to adjacent ependyma as well as subependymal tissues.[28] However, arguments were also put forward suggesting the apical exit of progeny JHMV from infected ependymal cells.

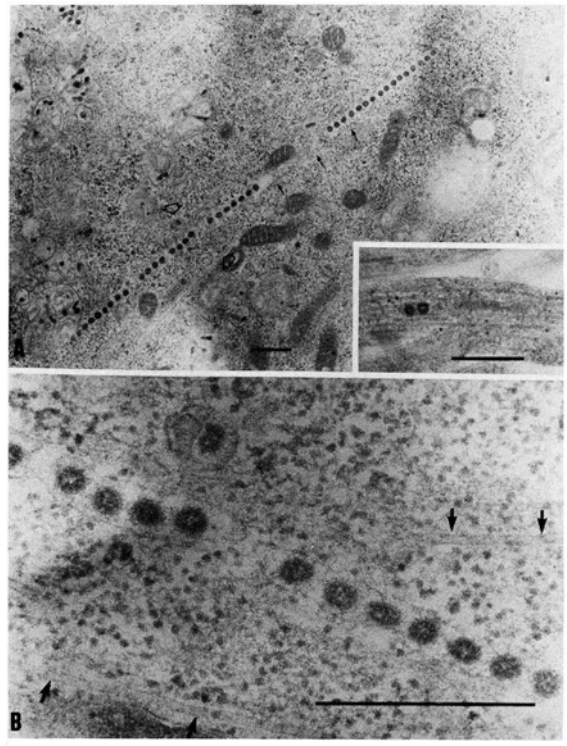

Figure 2. A demonstrates progeny JHM virions forming linear arrays within cisternae of infected OBL21 cells. The solid arrows point to an immediately adjacent microtubule. The inset shows two virions within a neurite from an infected hippocampal neuronal culture. B is a higher resolution image of the area bordered by the open arrows in A. The arrows in B point to microtubules. The bars in all panels represent 0.5 μm.

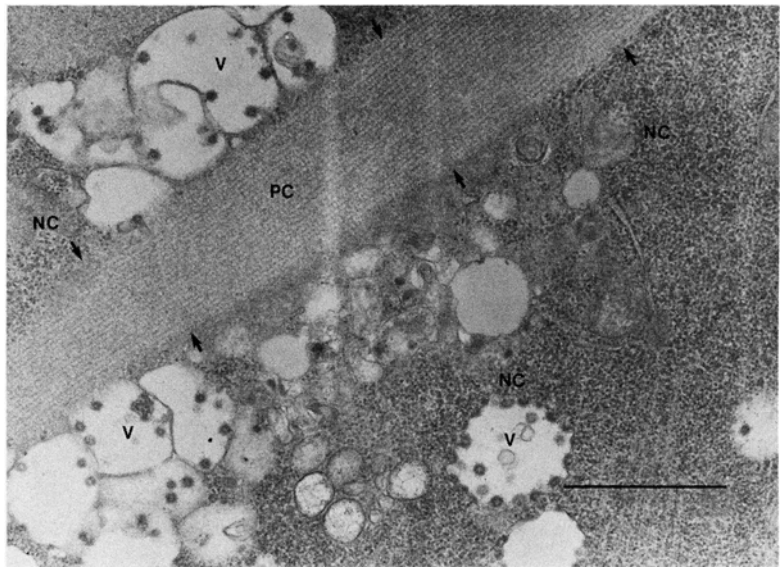

Figure 3. OBL 21 neuronal cell cultures inoculated with wtJHMV at a moi of 10 pfu/cell were treated with 10 μg/ml vinblastine sulphate at 36 hours post-inoculation then processed for electron microscopy 12 hours later. Arrows outline a tubulin paracrystal, PC, around which nucleocapsid core components, NC, and virions within vesicles, V, have closely associated. Bar represents 1 μm.

The pattern of distribution of N and S in hippocampal neurons reported here indicates that assembly and/or trafficking takes place somato-dendritically as observed previously[13,29] and in concordance with the reported presence of Golgi elements within dendrites.[30] The observed presence of both viral proteins in axonal-like neurites indicates one of two possibilities: assembly and/or trafficking may be nonpreferentially targeted toward both axons and dendrites or, viral proteins present within axons may result from transsynaptic transmission with subsequent retrograde spread to the cell soma. JHMV penetration by olfactory and trigeminal nerves implying transport in the anterograde direction, lends support to the former assumption,[3] although the latter while less likely, is also plausible. Of possible relevance to the above observations is the intriguing protein sequence homology between N and tau in which a respective 42% and 20% amino acid sequence similarity and identity exists. This was found by Dr. Michael Clarke of the Department of Microbiology and Immunology at UWO using the nucleotide sequence of N to search the Gen Bank data base. Optimal alignment of a putative tau microtubule binding motif was found between amino acids 328 and 340 inclusive within the carboxy-terminal portion of N.

To further assess the importance of microtubules in transporting newly assembled virions to the cell exterior, studies were carried out using the neuronal cell line OBL21. This cell line was clonally derived from CD.1 mouse olfactory bulb cultures immortilized with a replication-defective retrovirus vector carrying the avian myc gene.[15] The OBL21 line is a stable, homogeneous line expressing neurofilament but not glial fibrillary acidic protein and has maintained this phenotype while in our hands (data not shown). As with JHMV infected hippocampal neurons, assembly and/or trafficking of viral products N and S occurred in the neurite-like processes of OBL21 cells. In some infected cells mature virions were organized in precise linear arrays within cisternae adjacent to parallel microtubules (Fig. 2). To demonstrate virus-microtubule association more rigorously, infected OBL21 cultures were treated with vinblastine, a mitotic inhibitor which also inhibits axonal transport by promoting the depolymerization of microtubules and concomitant formation of tubulin paracrystals.[31] The resulting collapse of the microtubule framework was associated with the formation of massive aggregates of viral core components and membrane bound progeny around the tubulin paracrystals (Fig. 3).

In summary, neurons are targets for JHMV in vivo and in vitro. Variants characterized as having neuroattenuated phenotypes in vivo don't appear to possess altered tropism for primary hippocampal neurons in culture suggesting that the basis for in vivo neuroattenuation may be more complicated than initially thought. Trafficking of viral structural proteins in primary hippocampal neurons, as well as the involvement of microtubules in the movement of progeny virions in OBL21 cells, strengthens the supposition that rodent neurons may be capable of spreading the infection transneuronally.

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