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The murine coronavirus as a model of trafficking and assembly of viral proteins in neural tissue

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Infections of the central nervous system (CNS) by neurotropic viruses result in highly variable diseases and pathologies, depending on the agent involved. The outcome of the infectious process may be the consequence of both the replication strategy of the virus and the host's ability to control the infection and the neural cells that are targeted. Information about the dissemination or trafficking of virions and virus components within the neuronal, glial and other cells of the CNS is, therefore, essential for understanding the disease process.

Coronavirus diseases of the Central Nervous System

Much attention has recently been focused on the pathogenesis of virus-induced neurological diseases in rodent and primate model systems. Among the agents studied is the neurotropic murine J. Howard Mueller corona-

The replication of JHM, a murine coronavirus, provides a useful model of the assembly and dissemination of viral components in neuronal cells. Involvement of microtubules in virus trafficking is an important feature which may explain dissemination of the infection from primary cell targets at olfactory, hippocampal and cerebellar sites within the central nervous system, resulting in severe neuropathies.

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virus (JHM), which is capable of inducing CNS disease in susceptible rodents and monkeys^{1,2}. This virus-host model has been the focus of our attention. A spectrum of pathological processes is observed after intracranial inoculation of JHM virus (JHMV) into preweaning rats, ranging from acute, fulminant encephalitis to delayed onset and chronic demyelination^{2,3}. Previous studies have shown the nature of the disease process that predominates in rat pups inoculated intracranially to be a function of several host and viral deter-

minants including the strain of the animal used, post-natal age at the time of inoculation, length of time elapsing between inoculation and development of clinical signs, immunologic status of the host^{4,5} and variance in the molecular phenotype of the virion's major spike glycoprotein⁶⁻⁸.

With Wistar Furth rats, intracranial inoculation of JHMV during the first week of life invariably results in development of an acute encephalitis, whereas delay of the infection to the second week or later, up to the time of weaning, frequently results in a chronic, demyelinating disease⁴. The normal onset of resistance to JHMV which develops after weaning, can be overcome if the rats are treated with immunosuppressant drugs or are genetically athymic^{5,6}. These findings imply that the cellular immune response has a role in resistance. However, the disease that is provoked by JHMV in post-weaning, immunodeficient rodents is predominantly of the neuronal grey matter rather than of the white matter regions of the CNS⁶. The pathology that follows indicates the involvement of both immunologic and non-immunologic factors. Postnatal development of rat CNS involves maturation of the oligodendrocytes, during the first three weeks⁹. Oligodendroglial differentiation is evidently a determinant of the ensuing disease, depending on whether the neurons or neurons together with the myelin producing glial cells become infected.

Dissemination of infection

Coronaviruses can be disseminated to the CNS by transneuronal spread from peripheral sites. JHMV and strains of mouse hepatitis virus (MHV) such as A59 invade the CNS of mice following intranasal inoculation through a predominantly retrograde transneuronal route along the olfactory nerves^{10,11}. Consequently, interneuronal spread is thought to account for virus movement along this route into the CNS. Subsequent dissemination within the CNS to other regions was found to follow the path of specific neuronal tracts^{12,13}. Trafficking of viral components by immunohistochemical and *in situ* hybridization techniques revealed that when infecting mice, MHV-A59 manifests a tropism for the neurons of olfactory nuclei and neuronal clusters in specific regions of the fore-brain^{10,14}. In rats, JHMV appears to have a highly selective tropism for hippocampal and cerebellar Purkinje neurons (Fig. 1; Refs 15,16).

Because of the difficulties in carrying out studies on the pathogenic mechanisms of coronavirus infection in whole animals, experimentally amenable *in vitro* systems have been developed that rely on dissociated primary neural cell cultures derived from explants of neonatal hemispheres of rodents. Such cultures permit enrichment for specific neural cell types, including hippocampal neurons, astrocytes and oligodendroglia¹⁷. With the oligodendroglia from rats, it was shown that at specific stages of differentiation, cells of this lineage become conditionally permissive targets for JHMV, whereas cells of the lineage related to type-1 astrocytes can not be directly infected¹⁸. By contrast, there is no similar specificity in murine glial cell tropism for JHMV, for some unknown reason¹⁹. Additionally, observations have indicated that the process of *in vitro* differentiation of rat-derived oligodendrocytes, which matches accurately that occurring in the CNS²⁰, is a crucial determinant controlling JHMV replication and pathogenesis within white matter regions, and thus influences the development of demyelinating disease²¹.



Fig. 1. Detection of JHMV components in neurons and glia within the CNS of an acutely infected weanling rat, 6 d after intranasal inoculation. (a) Immunohistochemistry shows the N protein to be in the vicinity of the hippocampus. An arrow points towards a positive neuron. Some of the cells positive for N have the morphology typical of the oligodendroglia. (b) Viral RNA is evident in two neurons of the hippocampus (arrows) in an acutely infected pre-weanling rat. The RNA was demonstrated in an autoradiogram by means of *in situ* hybridization to a ³⁵S-labelled cDNA probe made against N of MHV. Scale bar = 30 μ m. Reproduced with permission from Ref. 16.

Virus assembly and dissemination

The general mechanisms by which viruses are assembled within, and exit from, their host cells is an important issue in determining the outcome of any virus-cell interaction. Since the patterns of synthesis of viral materials relate to those in the host cell, viruses can be used as probes for intracellular protein sorting and trafficking. Viruses have been particularly useful in cells with polarized membrane domains. For instance, vesicular stomatitis (VSV) and influenza viruses, which bud from polarized epithelial cells at the basolateral or the apical domains, respectively, or at the comparable somatodendritic or axonal domains of neurons²², do so as a consequence of targeting of their envelope glycoproteins

Table 1. Diseases, tropism and trafficking associated with representative neurotropic viruses

Virus type	Disease ^a	Infected cells in the CNS ^b	Intracellular distribution of antigen ^c	Axonal transport ^d	Refs
Borna disease	E, behavioural abnormalities	N, A, O, Ep	Nu, Ax, Sd	R/An	48,49
Measles	E, SSPE	N, Ep	Ax, Sd	R/An	47
Mouse hepatitis	E, demyelination	N, A, O, Ep	Ax, Sd	R/An	2,29,45,52
Herpes simplex type 1	E, M	N, A	Nu, Ax, Sd	R/An	32,38
Pseudorabies	E	N, A	Nu, Ax, Sd	R/An	39,40
Poliovirus	M, poliomyelitis	N	Ax, Sd	R/An	46
Rabies	E, paralytic rabies	N	Ax, Sd	R/An	33,34,42,43
Reo-type 3	E	N	Ax, Sd	R/An	35-37

^aE, encephalitis; M, meningitis; SSPE, subacute sclerosing panencephalitis.

^bN, neurons; A, astrocytes; O, oligodendrocytes; Ep, ependymal cells.

^cNu, nucleus; Ax, axonal; Sd, somatodendritic.

^dAn, anterograde; R, retrograde. Bold letter indicates the predominant direction of transport.

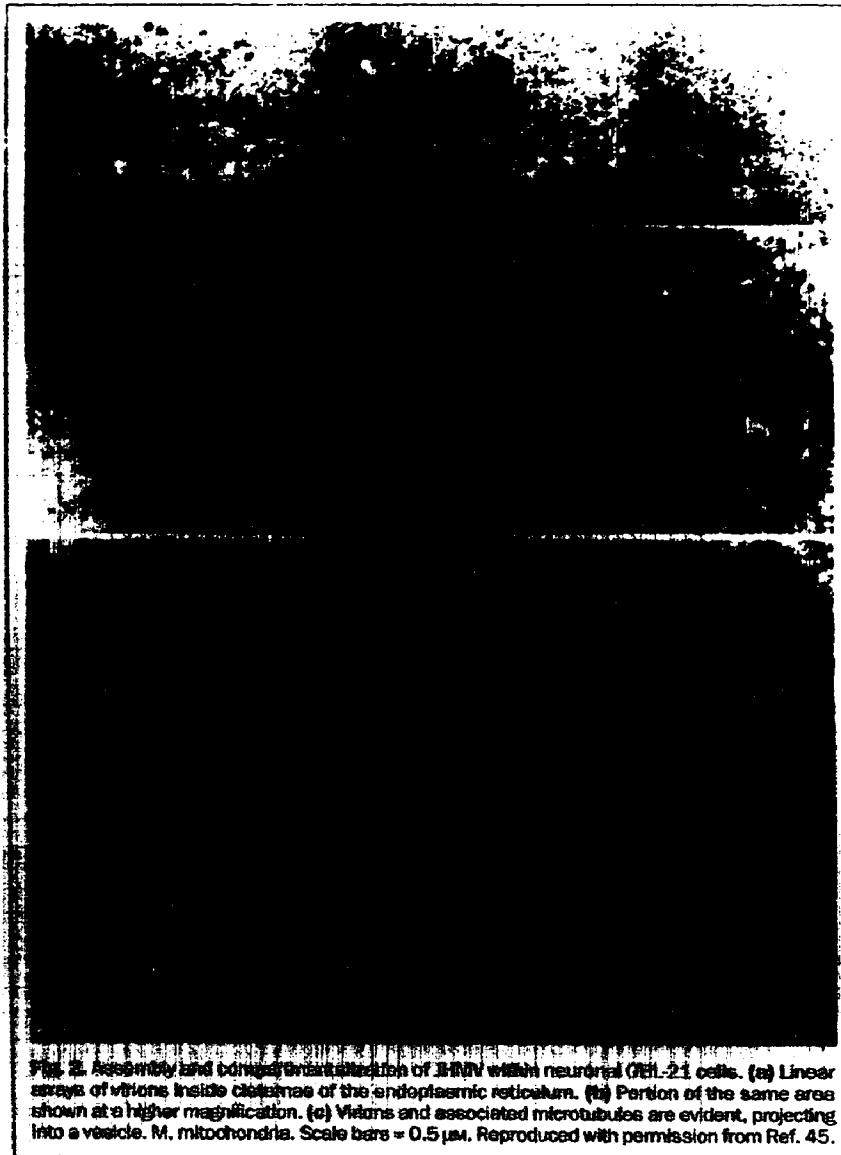


Fig. 2. Assembly and transport of JHMV within neuronal (A9L-21) cells. (a) Linear arrays of virions inside the lumen of the endoplasmic reticulum. **(b)** Portion of the same area shown at a higher magnification. **(c)** Virions and associated microtubules are evident, projecting into a vesicle. M, mitochondria. Scale bars = 0.5 μm. Reproduced with permission from Ref. 45.

towards specific membranous compartments²²⁻²⁴. Similarly, MHV was shown to mark the constitutive secretory pathway in AtT20, a murine pituitary tumor cell line, and provided insight into the site of sorting or divergence from the regulated secretory pathway in these cells²⁵. These types of studies provide us not only with an understanding about control over the secretory pathways in eukaryotic cells but also generate information applicable to viruses *per se*; for example, how viruses may interact with neurons to facilitate their spread throughout the CNS (see section on trafficking in neurons and Table 1, below).

Assembly of JHMV and MHV-A59 virions in fibroblastic cells occurs within the perinuclear region by budding at the transitional reticulum vesicles, a compartment positioned between the rough endoplasmic reticulum (RER) and Golgi apparatus²⁶⁻²⁸. The time and location of virion assembly appears to be controlled by the kinetics of synthesis and insertion of the integral membrane glycoprotein (M) into the membranes of these transitional vesicles^{26,28}. The assembly process of MHV seen in fibroblasts may be ubiquitous as shown with AtT20 murine pituitary tumor cells²⁷, and cultured mouse spinal cord neurons²⁹, in which progeny virions are disseminated via the constitutive rather than the regulated, or induced, exocytic pathway²⁵.

Trafficking in neurons

To understand how JHMV is assembled and released from neurons, one must consider the organization of these highly specialized, asymmetric host cells. Dendrites and axons, the two types of neurites emanating from neurons, perform specialized functions: the axons are involved

in conductance of electrical impulses away from the cell soma, while a multiplicity of dendrites receive and transmit signals to the cell exterior. Separation of impulse conductance either through axons or dendrites has been explained as a result of differences in their fine-structure, cytoskeleton and membrane protein constituents³⁰, implying that axons and dendrites are organized to carry out distinct functions because of their characteristic distribution of proteins and organelles³¹. Since the biosynthetic events taking place in neurons are generally confined to the somatodendritic domain, movement of materials to and from the extreme end of the apical (axonal) domain, which in large mammals can extend to over a meter in length, necessitates the existence of a highly efficient transport system. In fact, axonal transport is effected by two microtubule-dependent processes: fast transport of membranous organelles and slow transport of cytosolic proteins, especially those of the cytoskeleton^{30,31}.

Viruses of groups other than the Coronaviridae, including Herpes simplex type 1 (F.SV-1)³², rabies^{33,34} and reo-type 3 (Ref: 35-37), can invade the CNS by means of a rapid axonal movement which is presumably mediated by the microtubules (Table 1). HSV-1 (Ref. 38) and other herpes viruses, notably pseudorabies^{39,40}, are spread throughout the CNS by either a retrograde or anterograde transneuronal transfer process, depending on the virus involved. For example, rabies virus is moved predominantly in the retrograde direction^{33,41}, but can also be transferred by an anterograde pathway^{34,42}. Neurotropic reoviruses, among them reo-3, can gain access bidirectionally into the CNS by the transneuronal route³⁵⁻³⁷. The interneuronal progress of the above agents towards and within the CNS can be interrupted by drugs affecting microtubule integrity, such as colchicine, nocodazole and vinblastine sulphate^{37,43-45}. It has not been established whether the microtubules are also involved with viruses such as poliovirus⁴⁶, measles⁴⁷, pseudorabies^{39,40}, and the Borna disease agent^{48,49}, which invade and spread within the CNS by a specific transneuronal movement.

With regard to the JHMV-neuronal interaction, the disposition of viral components and progeny virions observed within primary rat hippocampal neurons and OBL21 cells fits the idea that infection within the CNS is spread transneurally (Fig. 2). Judging by the pattern of virus spread to adjacent ependyma and subependymal tissues of the CNS, the process by which JHMV is externalized appears to involve the release of virions from polarized ependymal cells at their baso-lateral surface, although exit at the apical surface has not been ruled out⁵⁰.

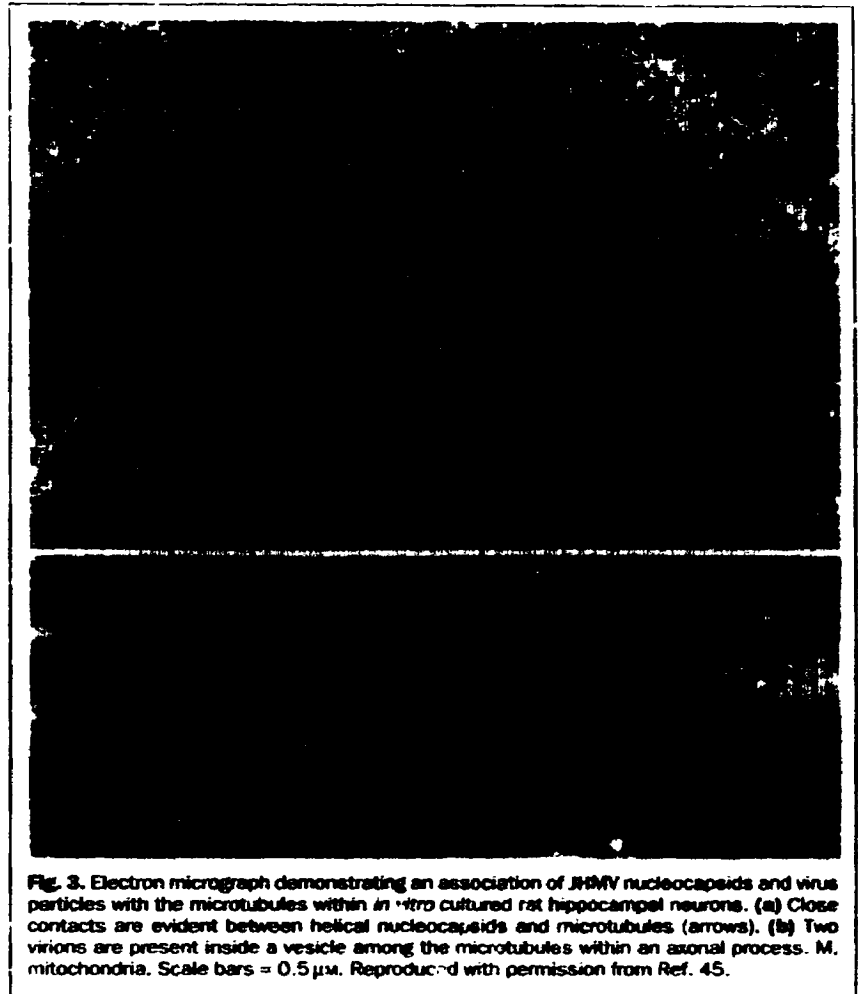


Fig. 3. Electron micrograph demonstrating an association of JHMV nucleocapsids and virus particles with the microtubules within *in vitro* cultured rat hippocampal neurons. (a) Close contacts are evident between helical nucleocapsids and microtubules (arrows). (b) Two virions are present inside a vesicle among the microtubules within an axonal process. M, mitochondria. Scale bars = 0.5 μ m. Reproduced with permission from Ref. 45.

The distribution of the nucleocapsid (N) and spike (S) components of JHMV in hippocampal neurons occurs in a pattern that suggests that trafficking may occur somatodendritically⁵², as has been shown for explanted neurons of mice^{20,51,52}. With JHMV, vectorial movement is likely to be related to the location of vesicles of the Golgi apparatus within the soma and dendrites³¹, which are involved in coronavirus assembly and transport^{25,26} (Figs 2 and 3). Microtubule-associated proteins (MAP), among these tau, play a significant role in the organization of the axonal microtubules^{30,31}. Codistribution of the viral N, S components and tau within axons or very long neurites with axonal morphology (Fig. 4) further implies the participation of axonal trafficking in propelling JHMV particles along neurons. As indicated in Table 1, evidence of coronavirus dissemination at the basolateral and/or apical domains of neurons contrasts with the selective release of other enveloped viruses from only one domain of polarized cells²⁷.

Evidence of an association between N and neuronal microtubules *in vitro*^{45,52} (Figs 3 and 4) is another instance of intimate contacts which has been previously

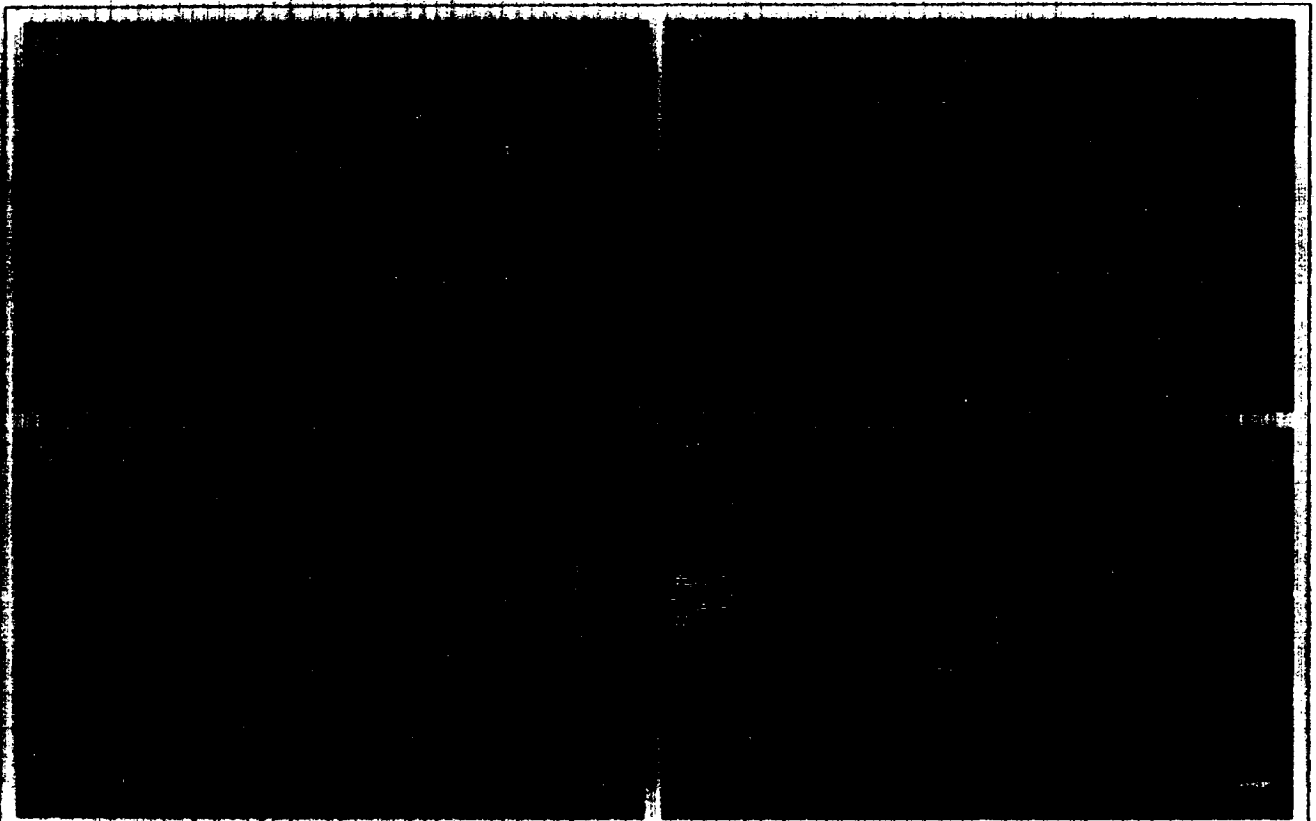


Fig. 4. Localization of JHMV proteins in neuronal cell domains. Cultured rat hippocampal neurons were challenged with virus on the 20th day following explantation and then fixed and stained 1 d after inoculation. (a) Neurons reacted with antibodies against N followed by secondary antibodies conjugated to FITC. (b) The same neurons stained with antibodies against tau, then secondary antibodies conjugated to Texas Red. (c) Staining of S protein with anti-S antibodies as in (a). (d) The same neurons as in (c) reacted to label tau. Arrows point towards prominent neurites (axons?), positive for both JHMV and tau. Note the paucity or absence of tau in the shorter (dendrites?) neurites. Scale bars = 10 μ m (all parts). Reproduced with permission from Ref. 45.

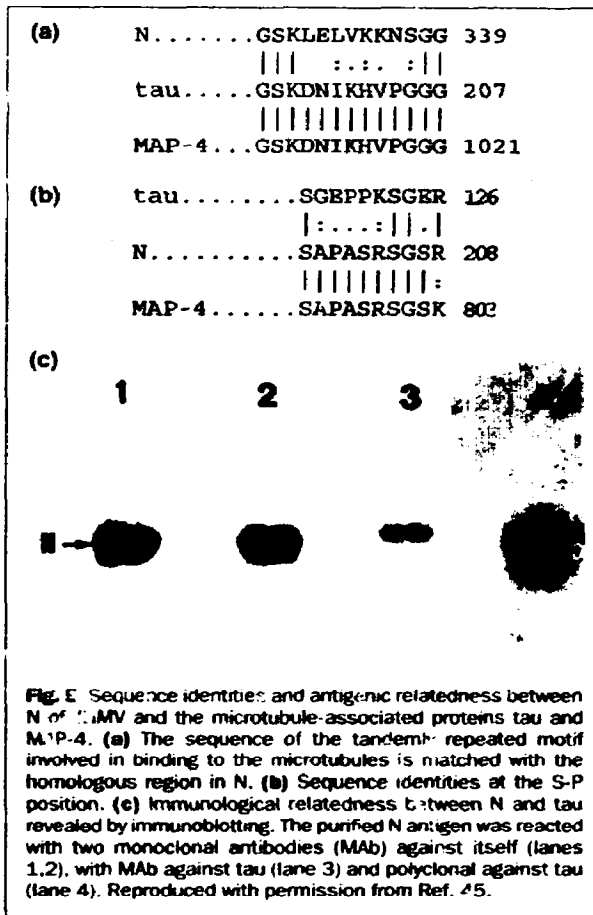
demonstrated with reovirus⁵³ and adenovirus⁵⁴. The observed association of N with microtubules is most probably not fortuitous but is a reflection of sequence similarities between N and the microtubule-binding motif of tau (Fig. 5a). This draws attention to the evolution of viruses as parasites. By exploiting the host's structure-function characteristics, the viral evolutionary homologue can mimic a fundamental cell process. The tau-microtubule linkage is established through a stretch of 18 amino acid residues tandemly repeated three to four times at the carboxy-terminal end of tau⁵⁵. When constructed as an isolated peptide, this 18-mer can bind to microtubules⁵⁶. Within the tau repeated se-

quence, 12 of the 18 invariant residues could be aligned optimally with residues 328-340 of N (Fig. 5a), which is consistent with the presumed similarity of microtubule attachment sites in N and tau. It is remarkable that an identical microtubule binding domain was uncovered by a computer search in another cytoskeletal component, the MAP-4 protein (Fig. 5a). Another region of sequence similarity between tau and MAP-4 exists in the serine/proline-rich domain⁵⁷, and this stretch also has relatedness to residues 199-208 of N (Fig. 5b). In this context, it is worth noting that codistribution of Sendai virus proteins and the MAP-2 component can be recognized immunocytochemically at somatodendritic sites within infected neurons⁵⁸. Immunological recognition of N by anti-tau antibodies (Fig. 5c), supports the biological relevance of the sequence similarities.

Interaction between N and microtubules could have implications for the assembly of progeny virions. When free molecules of N in the cytosol become attached to microtubule surfaces, they will be in the correct position for any subsequent association with the integral coronavirus membrane glycoprotein M prior to budding into ER vacuoles. Such an assembly sequence is consistent with the observed distribution of virions

Questions for future research

- Are microtubules and other cytoskeletal elements involved in the externalization of the progeny from their intracellular sites of assembly?
- Are progeny disseminated via axonal and/or dendritic transfer?
- From which point on the neuronal surface (e.g. the synapse) does transneuronal virus spread occur?
- Are motor proteins (e.g. dynein, kinesin) involved in the transport?



inside membranous cisternae of neuronal cells in which the presence of adjacent microtubules is evident (Fig. 2). In contrast, when they are produced in excess and fail to become incorporated into progeny virions, the nucleocapsids containing N perhaps become transferred into the neurites by slow axonal transport^{30,31}. These observations support the notion of an association between nucleocapsids and the microtubules of neurites⁵² (Fig. 3). Future elucidation of these phenomena is likely to be significant for our understanding of cell and viral protein trafficking and virus-induced neuropathogenesis.

Acknowledgements

Supported by the Medical Research Council of Canada (MRC) and a studentship to K.K. from the Multiple Sclerosis Society of Canada.

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