Ultrastructural description of rabies virus infection in cultured sensory neurons

Myriam L Velandia*, Rosalía Pérez-Castro*, Hernán Hurtado*/**, Jaime E Castellanos/+

Instituto de Virología, Universidad El Bosque, Transv. 9A Bis 132–55, Edificio de Rectoría, Laboratorio 205, Bogotá, Colombia *Laboratorio de Neurociencias, Instituto Nacional de Salud, Bogotá, Colombia **Facultad de Biología, Universidad Militar Nueva Granada, Bogotá, Colombia

Primary cultures were made from adult mouse spinal ganglia for depicting an ultrastructural description of rabies virus (RABV) infection in adult mouse sensory neuron cultures; they were infected with rabies virus for 24, 36, and 48 h. The monolayers were processed for transmission electron microscopy and immunochemistry studies at the end of each period. As previously reported, sensory neurons showed great susceptibility to infection by RABV; however, in none of the periods evaluated were assembled virions observed in the cytoplasm or seen to be associated with the cytoplasmic membrane. Instead, fibril matrices of aggregated ribonucleoprotein were detected in the cytoplasm. When infected culture lysate were inoculated into normal animals via intracerebral route it was observed that these animals developed clinical symptoms characteristic of infection and transmission electron microscopy revealed assembled virions in the cerebral cortex and other areas of the brain. Sensory neurons infected in vitro by RABV produced a large amount of unassembled viral ribonucleoprotein. However, this intracellular material was able to produce infection and virions on being intra-cerebrally inoculated. It can thus be suggested that the lack of intracellular assembly in sensory neurons forms part of an efficient dissemination strategy.

Key words: rabies virus - sensory neurons - viral assembly

Rabies virus (RABV) is a negative, non-segmented, single-strand RNA virus which infects animals and the human nervous system (Mattos et al. 2001). RABV entry to cells is due to viral glycoprotein (G) binding to some of the membrane proteins proposed as being viral receptors (the nicotinic acetylcholine receptor, the neural cell adhesion molecule, and the p75 low affinity neurotrophin receptor) (Lafon 2005). Once within the cytoplasm, the ribonucleoprotein (RNP) complex is released; this is formed by the RNA strand and non-structural proteins N, P, and L (nucleoprotein, phosphoprotein, and viral polymerase, respectively). Viral transcription and replication begin on this complex, following processes culminating in new viral progeny being released (Finke & Conzelmann 2005). Viral capture and transport in a rabid accident is produced by endocytic process, reaching the soma of sensory neurons located in the spinal ganglia or the motoneurons of the spinal cord's (SC) ventral horn. Sensory neurons' ability to capture, transport, and replicate RABV has been described in sensory neuron cultures from different animal species. Our group has developed an in vitro RABV infection model using adult mouse sensory neurons which has confirmed and evaluated susceptibility to infection and the antiviral and anti-replication effect of some drugs on these cells (Castellanos et al. 2005). Such susceptibility has been observed in models in vivo and it has been confirmed that these neurons are fundamentally involved in viral transport from the periphery to the central nervous system (CNS) via retrograde and anterograde axonal transport (Tsiang et al. 1983, 1989, 1991, Coulon et al. 1989, Dietzschold et al. 2005).

Even though the virus' tropism towards neurons is widely known, it has been observed that some non-neuron cells may support viral entry and replication. Laboratory-adapted "fixed strain" viruses are frequently used in in vitro studies for infecting cells or tissue having different origin to the neurons. It has been found that baby hamster kidney fibroblast cells present high susceptibility to infection and produce assembled viral particles, recuperating in culture medium 12 to 18 h post-infection (pi) (Madore & England, 1977). On the contrary, even though high susceptibility to infection has been observed in myocyte primary cultures, no assembled viral particles have been detected or released to the extracellular space. Just a large amount of viral inclusions can be observed in cytoplasm, even up to 72 h pi (Tsiang et al. 1986).

On the other hand, it has been observed in both in vitro and in vivo neuron infection studies that even though marked viral neurotropism is maintained, there are differences in infection ability and viral production between different neuron types. Sensory neurons pro-

duce between 10- to 100-fold less viral particles respecting cortical neurons (Mrak & Young 1994). Such results suggest that each neuronal type has particular metabolic and biochemical characteristics which may interfere in different ways in the main processes of the viral cycle, just the same as that observed in fibroblasts and myocytes. It is thus particularly interesting to evaluate the ultrastructural characteristics of RABV infection in sensory neuron cultures and adult mouse cortex neurons by immunochemistry [immunoperoxidase (IP), immunofluorescence (IF), and transmission electron microscopy (TEM)] techniques.

MATERIALS AND METHODS

Sensory neuron cultures - Four independent adult ICR strain mouse spinal ganglia sensory neuron cultures were done according to previously reported protocol (Castellanos & Hurtado 1999). The animals were sacrificed by cervical dislocation; the complete vertebral column was extracted and the ganglia dissected. They were then mechanically and enzymatically dissociated in a 200 U/ml collagenase solution in complete medium (CM, DMEM supplemented with 10% bovine foetal serum, 100 U/ml penicillin, and 100 μg/ml streptomycin). The cell suspension was centrifuged at $200 \times g$ for 5 min. Cell viability was then evaluated with Trypan blue and 2500 cells seeded on Thermanox coverslips pre-treated with poly-L-lysine (10 μg/ml) in 24-well dishes and kept at 37ºC with 5% CO_2. The medium was changed each 48 h.

In vitro infection - Sensory neuron cultures (consisting of sensory neurons, Schwann cells, and fibroblasts) were infected with a 1:10 dilution of CVS-11 strain rabies virus (Challenge Virus Standard), produced in mouse brain ($10^{2.43}$ DL_{50}) diluted in complete medium. The inoculum was removed 1 h pi, complete medium was introduced and the cultures were taken to 24, 36, and 48 h pi. Once these periods had elapsed, half of the infected cultures were fixed with 4% paraformaldehyde in PBS, pH 7.4, for later processing by IF. The rest of the samples were processed by IP and then fixed with Karnowsky's modified solution (0.5% glutaraldehyde, 2% paraformaldhyde in 0.1M phosphate buffer, pH 7.4). A total of four cultures were processed with three replicas per culture.

In vivo infection - Three independent sensory neuron cultures were processed; they were infected at 120 h pi (according to the previously described protocol) and lysed in CM by mechanical pipetting. This cell lysate was then inoculated by intracerebral route into five ICR adult mice; these animals were observed daily and sacrificed on day 5. Six-week-old ICR mice were used as infection controls and intra-cerebrally inoculated with a 1/10 dilution of CVS-11 strain RABV and the mock-infected mice were inoculated with CM. Both groups of animals were anaesthetised with a mixture of ketamine (90 mg/kg) and xylazine (15 mg/kg) on the fourth day pi. They were then intra-cardially perfused with a washing solution (0.8 % NaCl, 0.1% procaine, 0.1% glucose) and fixed by perfusion with modified Karnowsky's solution. The brain and cerebellum were extracted and each hemi-sphere was independently processed for later immunochemistry (IF and IP) or TEM. Tissue processed by immunochemistry was post-fixed in the same fixing solution overnight at 4ºC and then vibratome sectioned into 100 μm thick sections (Castellanos et al. 1996). All the procedures described above had been previously approved by the Instituto Nacional de Salud's Ethics' Committee, in line with Colombian Ministry of Health Resolution 8430/1993.

Immunochemistry - The samples (cells or tissue) were permeabilised with 50% ethanol. Endogenous peroxidase was inactivated by using a 0.05% NaN_3 and 0.04% H_2O_2 solution in PBS. Non-specific binding sites were blocked with 10% horse serum. Viral antigen was detected by using a hamster anti-rabies antibody at 1:900 dilution (diagnostic antibody produced by the Instituto Nacional de Salud, Colombia) incubated in Tris buffer, pH 8.6, for 1 h at 37ºC (Castellanos et al. 1996). The respective biotinylated anti-hamster secondary antibody was then added (2.5 μg/ml). Samples were then incubated with biotinylated avidin-peroxidase (ABC Vector Inc) complex diluted 1:100, a 0.05% diaminobenzidine solution in 50 mM HCl Tris buffer pH 7.2 and 0.02% H_2O_2 used as chromogen. The reaction was interrupted by successive washing with tap water. Sensory neuron cultures fixed with PFA were processed by IF. They were thus permeabilised with Triton X-100 and incubated with hamster anti-rabies antibody coupled to FITC (1:30) in PBS at 37°C for 30 min in a wet chamber. The cells were then counterstained with Evans' blue (1/10.000) and washed with PBS and distilled water. The cells were then mounted in aqueous medium (buffered glycerine, pH 9.0) and observed by epifluorescence microscopy.

Electron microscopy - Once immunoperoxidase developing time had elapsed; three samples by duplicate per culture were processed for TEM. Both kinds of sample were then post-fixed with 1% osmium tetroxide and dehydrated in increasing ethanol gradients (70, 80, 90, 95, and 100%). The monolayers were infiltrated with a mixture of 100% ethanol and Epon-812 Polybed resin in 3:1, 1:1, 1:3 ratios and then embedded in a mixture of Epon-Araldite resins. Slices of nerve tissue were infiltrated with a mixture of propylene oxide and Epon-Araldite resin in the ratios described above. They were finally embedded in Epon-Araldite and polymerised at 67ºC for 18 h. Semi-thin and ultra-thin sections were obtained by using a Sorvall MT1 ultramicrotome. Semi-thin sections were stained with toluidine blue and specific areas were defined for obtaining the ultra-thin sections. These were contrasted with uranyl acetate and lead citrate and observed using a Carl Zeiss EM 109 electron microscope.

Infected cell count - Two replicas of each culture were taken 48 h pi and processed by immunoperoxidase. Infected neuronal and non-neuronal cells were counted under a microscope; the proportions so obtained were submitted to angular transformation and analysed by one-way ANOVA and Newman-Keuls' multiple mean comparison test. There was considered significant p values less of 0,05.

RESULTS

Morphological description - The sensory neurons cultures presented a typical heterogeneous aspect. Neuronal and non-neuronal cells were identified. The neurons were seen to be refringent, spherical, having an eccentric nucleus; neuritic processes were observed in most of them which increased in length during culture time. The non-neuronal cell population (Schwann cells and fibroblasts) appeared to be non-refringent cells which were strongly adhered to the surface of the plate. No appreciable differences were observed between infected culture morphology compared to that of non-infected ones. When evaluating the number of neurons and non-neuronal cells infected with RABV at 24 h pi, it was observed that 39.8% of the neurons had become infected whilst the population of non-neuronal cells (fibroblasts and Schwann cells) in cultures presented 2.1% infection (Table).

TABLE

Percentage of neuronal and non-neuronal cells from sensory neuron cultures infected with rabies virus

	Infected (%)	Non Infected (%)	Total (%)
Neurons	1035 (39.8)	1568 (60.2)	2603 (100)
Non-neuronal	222 (2.1)	9968 (97.8)	10,190 (100)
Total	1257	11536	12,793

Immunochemistry - Viral antigen was observed in sensory neuron cultures at three periods pi, evaluated by both IF and IP techniques. Immunoreactivity (IR) was low at 24 h pi and was located mainly in the perinuclear region and in sensory neuron neurites. IR was also observed in some non-neuronal cells. A large amount of cytoplasmic vesicles which were IR for viral antigen 36 and 48 h pi were observed in the cytoplasm of infected neurons; these vesicles were located in the cytoplasm's central area. An increase in the number of these vesicles was also observed 48 h pi; they were located very close to the neuronal membrane (Fig. 1A). Infection in the brain was observed in different areas of the cortex and in the hippocampus' pyramidal cell layer. IR was observed in the cerebellum in the molecular and granular layers of the cortex. However, it was preferentially detected in Purkinje neuron soma in which cytoplasmic inclusions were identified close to the cell membrane (Fig. 1B).

Infected cell ultrastructure - Infected neuron cytoplasm presented multi-vesicular and lamellar bodies. It was possible to identify subcellular structures such as lysosomes, lipofuscin granules, mitochondria, and Golgi apparatus. Type B neurons (dark neurons) were most found in cultures. Cytoskeleton structures, such as microtubules and neurofilaments, could only be identified in some neurons (i.e. being more evident in neuritic processes). Granular electron-dense aggregates (fibrillar matrices) were observed during all pi periods around the nucleus or very close to the plasma membrane. Such aggregates were more evident at 36 and 48 h pi (Figs 2A, B). TEM revealed that these electron-dense structures were half-moon shaped and were IR for viral antigens. This morphological pattern was maintained during the periods analyzed; however, a larger number of these aggregates was found at 48 h pi, located very close to the plasma membrane or fused to it (Figs 2C, D). No assembled viral particles were found at any time analyzed or in the cytoplasm or close to the cell membrane. Aspect of mock infected sensory neuron cultures is showed in Fig 2E.

Infected cell lysates were inoculated by the intracerebral route into mice at 48 h pi to confirm the infectious ability of fibril matrices present in the sensory neuron culture cytoplasm. The animals presented symptoms characteristic of RABV infection 5 days pi. The sections presented a positive reaction in the hippocampus, cerebral cortex, and the cerebellar Purkinje neurons when evaluated by IFA and/or IP. TEM revealed viral particles in pyramidal cells from the hippocampus, some of them presenting degeneration and dilated mitochondrial matrix. Each infected neuron cell presented 2 to 8 cytoplasmic inclusions which seemed to be Negri body-like (viral RNP) structures, observed as being a strongly electron-dense matrix; 75 nm diameter, variable length (approximately 200 nm), assembled viral
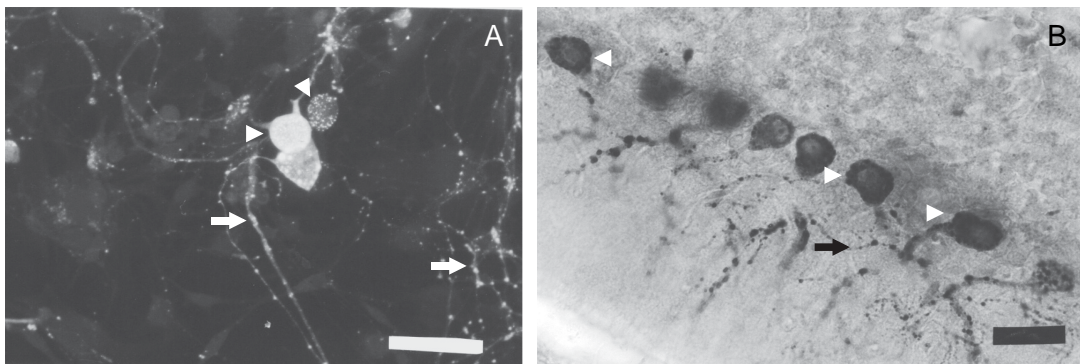

Fig. 1: immunochemical staining of rabies virus. A: sensory neurons culture processed by immunofluorescence. Infected sensory neurons (arrowheads); note the immunoreactive neurites (arrows); B: cerebellum sections processed by immunoperoxidase technique. Purkinje cell bodies are positive to viral antigen (arrowheads) and dendrites are shown with immunoreactive vesicles (arrow). Bars = 50 μm.
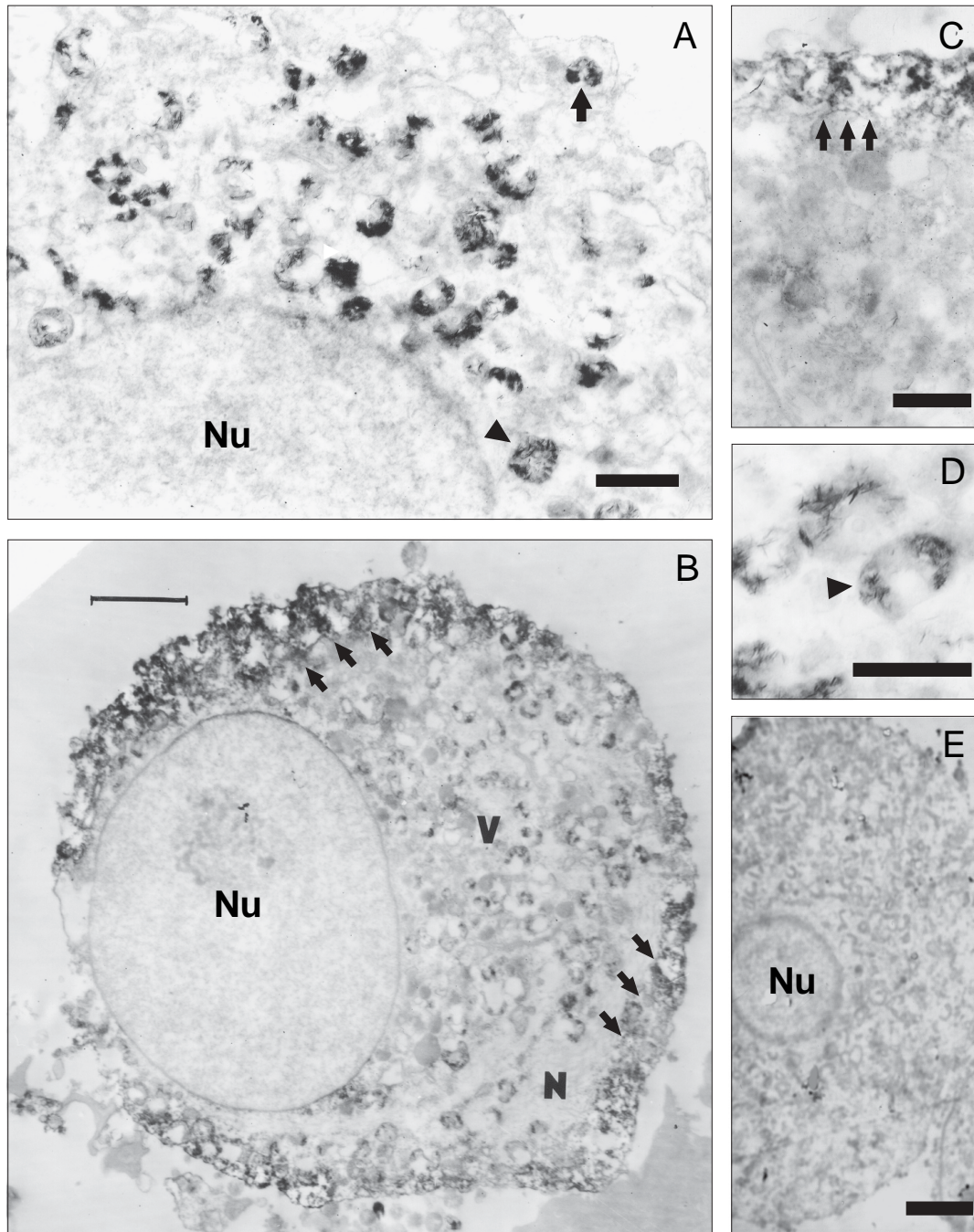
Fig. 2: electron micrographs of cultured sensory neurons. A: infected neuron 36 h pi. Many viral inclusions (arrow and arrowhead) were detected by using immunoperoxidase inside the cytoplasm near the nucleus (Nu). Bar = 1 μm; B: infected sensory neuron 48 h pi; viral inclusions (V) are concentrated near the plasma membrane (arrowhead). Bar = 5 μm; C: detail of cytoplasmic immunoreactive vesicles (arrows) and membrane fusion. Bar = 1 μm. D: detail of immunoreactive viral inclusion (arrows); note the electron-dense diaminobenzidine crystals. Bar = 0.5 μm. E: electron micrograph of sensory neuron from a non-infected culture; Bar = 1 μm.

particles were observed around such structures. These viral particles were also observed to be sometimes associated with vesicles close to the endoplasmic reticule (ER) or Golgi apparatus; they were only observed in these neuron cells budding through the cytoplasmic membrane of soma or some synaptic buttons. Electron immunomicroscopy revealed granular, rounded precipitates associated with ER. Assembled viral particles were observed close to these structures in some cases (Fig. 3).

DISCUSSION

Sensory ganglia are peripheral nervous system structures, mainly consisting of pseudomonopolar neurons and support tissue provided by Schwann cells and fibroblasts. Sensory neurons have been described and characterised both in vivo and in vitro according to their physiology, biochemistry, and morphology (Thomas et al. 1993). Ultrastructurally, and according to their density and ER distribution in the cytoplasm, these cells
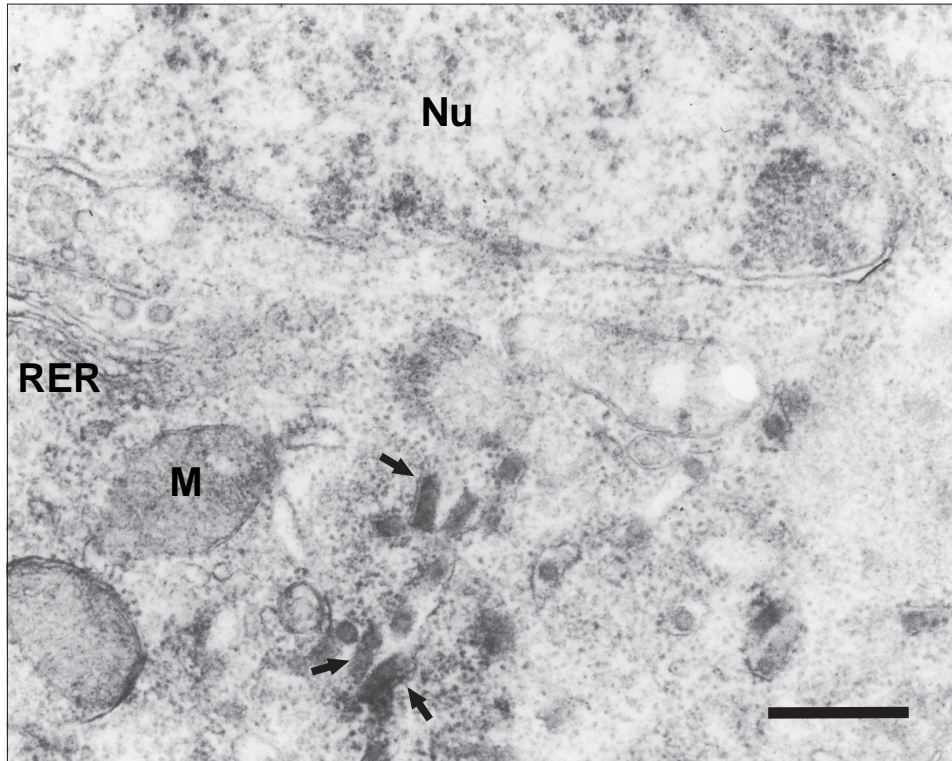
Fig. 3: electron micrograph of a cortex neuron from an infected mouse which was inoculated with infected sensory neuron cells lysate. Arrowheads point to assembled rabies virions inside the cytoplasm. Mitochondria (M), cell nucleus (Nu), and rough endoplasmic reticulum (ER) can also be seen. Bar = 0.5 μm.

can be classified into clear (type A) and dark neurons (type B) (Martinez et al. 2002). Our culture model favoured the presence of type B neurons, corresponding to intermediate and small nociceptive-associated neurons. On the other hand, the loss of large neurons (type A) could have been due to the technique employed for obtaining them and the culture's senescence. These findings coincide with those previously described by Sommer et al. (1986) in in situ models and by Martínez et al. (2002) and Rambourg et al. (1983) in in vitro models.

Viral antigen distributed in infected sensory neurons' soma and neurites was detected during the present work, confirming marked RABV neurotropism. High rabies' virus neurotropism was confirmed by the percentage of infected cells present in cultures where 39.8% of sensory neurons were infected, whilst only 2.1% of non-neuronal cells were infected.

A particular cytoplasmic IR structure was observed near the nucleus, associated or not with ER during all evaluated periods in infected neurons. These aggregates were dense fibril structures, suggesting that they were viral ribonucleoprotein (RNP), being free inside the cytoplasm or accumulated forming irregular-shaped structures. The absence of assembled viral particles in these cell cultures coincided with that preliminarily reported in rat sensory neurons and myocytes (Tsiang et al. 1986, Martinez et al. 2000). Both cell types can be considered to be primary viral capture and replication sites, suggesting that the virus initially enters these cells to avoid immune system attack and/or undergo some adaptation pro-

cesses (Iwasaki et al. 1973, Watson et al. 1981, Lycke & Tsiang 1987). Low assembled virus production in these types of cell could possibly be attributed to differences in the machinery of neurons from the sensory neurons and brain, these being transport and immune response evasion strategies.

The cells' biochemical and metabolic differences could be implicated in viral replication and transcription; they could also modulate interaction between viral and cellular proteins, concluding in correct viral assembly (Nakamichi et al. 2005). Rhabdovirus assembly depends on the correct interaction of RNP with protein M and that of the latter with the plasma membrane. A direct interaction between RABV matrix protein N-terminal region L domain and the WW domain present in the proteasome-associated membrane protein cytoplasmic region has been previously demonstrated (Charlton & Casey 1979, Lenard 1999, Mebatsion et al. 1999, Harty et al. 1999, Harty et al. 2001). It is possible that alterations are presented in the proteasome-associated membrane proteins domains' sensory neurons, thereby impeding correct M interaction with these neurons' membrane.

Regarding a transport strategy, the same line of thought as that reported by Mc Quaid et al. (1998) in neuronal models of infection with measles' virus could be adopted. They observed a large amount of viral nucleocapsids accumulating without assembled particles being produced in the syncytia formed during infection by the virus in Vero cells, suggesting that the measles virus uses different forms of dispersion, without the need to as-

semble viral particles. Viral dispersion and immune evasion would thereby become increased. It is possible that RABV may not be assembled in typical viral particles within the cytoplasm of sensory neurons by as yet unknown biochemical or metabolic mechanisms. By contrast, it may produce a large amount of RNP aggregates, these being constantly stored and transported during prolonged infection; such mechanism thereby guaranteeing the efficiency of spreading, silencing and persistence of infection in sensory neurons. These neurons could then transport RNP complexes to the CNS where the virus could conventionally assemble and disperse itself by using genetic information and a different cellular machinery. The latter may be suggested as intra-cerebrally inoculating supernatants and homogenised products from sensory neurons cultures into adult mice has led to the animals developing symptoms characteristic of infection. Assembled viral particles have also been observed in cortical neurons.

Our results suggest that sensory neurons support infection and offer RABV an excellent scenario for viral replication and transcription; however, a large amount of non-assembled RNP are produced which are able to produce the characteristic signs of paralysis and death in infected animals due to their biochemical and functional characteristics. These RNP also produce viral cortical neuron progeny which are assembled and released. Such phenomenon offers a strategy for the mass and silent production of viral RNP in sensory neurons which might be transported and then released in the dorsal horn of spinal cord so that RABV can then start on a new infectious cycle. The virus might possibly encounter suitable metabolic, biochemical, and functional conditions in the latter neurons, allowing correct and efficient assembly of new viral progeny. The present evidence leads to suggesting that RABV adopts different mechanisms in each type of neuron which it infects to increase its ability to disperse itself within the organism and avoid the immune system's attack. How and why the virus uses such types of strategy is still to be determined. Further studies are also needed for a much more precise definition of the sensory neurons' role during viral capture, transport, and spreading within the nervous system.

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