Unusual ultrastructural findings in dendrites of pyramidal neurons in the cerebral cortex of rabies-infected mice

Orlando Torres-Fernández, Jeison Monroy-Gómez, Ladys E Sarmiento

Previous studies using the Golgi technique have demonstrated alterations in the dendritic morphology of pyramidal neurons of the cerebral cortex of mice inoculated with the rabies virus. However, knowledge about the fine structure of dendrites in rabies infection is scarce. This work had the aim of studying the ultrastructure of dendrites in cortical pyramidal neurons of rabies-infected mice. Mice were inoculated intramuscularly with a street rabies virus of canine origin. The animals that showed an advanced stage of disease were fixed by perfusion with glutaraldehyde and paraformaldehyde. Brains were removed and cut on a vibratome to obtain coronal slices of 200 micrometers of thickness. Vibratome slices were subjected to the following treatment: postfixation, dehydration, embedding in epoxy resin and polymerization between glass slides. Ultrathin sections of oriented tissue fragments from the cerebral cortex were obtained and observed under electron microscope. The most significant ultrastructural findings were located within distal dendrites of cortical pyramidal neurons: loss of mitochondria, disorganization and loss of microtubules, formation of vacuoles interrupting the continuity of the cytoplasm and formation of myelin-like figures. These strange myelin figures, which apparently had not been reported in previous studies of rabies, were the most noticeable ultrastructural feature. They also differ from the best known myelin figures formed by concentric lamellae. The possible origin of these myelin figures as result of mitochondrial degeneration is discussed.

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UNUSUAL ULTRASTRUCTURAL FINDINGS IN DENDRITES OF PYRAMIDAL NEURONS IN THE CEREBRAL CORTEX OF RABIES-INFECTED MICE

Orlando Torres-Fernández*, Jeison Monroy-Gómez, Ladys E. Sarmiento

Laboratorio de Microscopía, Grupo de Morfología Celular, Instituto Nacional de Salud (INS), Av. Calle 26, No. 51-20, Bogotá-Colombia.

*Corresponding Author:

Adress: Laboratorio de Microscopía, Grupo de Morfología Celular, Instituto Nacional de Salud (INS), Av. Calle 26, No. 51-20, Bogotá-Colombia.
Tel: 571-2207700 ext. 1262
E-mail: otorresf@ins.gov.co

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Introduction

Rabies is an infectious disease caused by a neurotropic virus that mostly affects the central nervous system. The viral particles are inoculated into muscle fibers through the bite of rabies-infected animals. The virus enters through the neuromuscular junction and is lead to the spinal cord and then to the brain by retrograde axonal transport (Ugolini, 2011). In previous observations, we found evidence that the rabies virus entered the cerebral cortex through the pyramidal cells of layer V when mice were inoculated in their hind limb muscles (Lamprea & Torres-Fernández, 2008). In addition, the vulnerability of cortical pyramidal neurons infected with rabies virus was highlighted by a Golgi technique study that revealed significant alterations in dendritic morphology induced by both fixed and street rabies viruses (Torres-Fernández, Yepes & Gómez, 2007). Other authors have also found evidence of dendritic damage caused by rabies using different techniques (Li, Sarmento & Fu, 2005; Scott et al., 2008; Song et al., 2013).

While electron microscopic studies of nerve tissue infected with rabies are numerous, they rarely make specific reference to the ultrastructure of the dendritic tree of the neurons most frequently infected with rabies virus. The ultrastructural analysis of nerve cells and brain tissue infected with rabies has focused more on the location and description of viral particles and Negri bodies (Matsumoto, 1963; Hummeler, Koprowski & Wiktor, 1967; Miyamoto & Matsumoto, 1967; Murphy, 1975; Matsumoto, 1975; Hummeler & Atanasiu, 1996), and on the cell-to-cell virus transmission mechanisms (Schneider, 1975; Iwasaki & Clark, 1975; Iwasaki et al., 1985; Velandia et al., 2007). This work was carried out with the purpose of studying the fine structure of dendrites of cortical pyramidal neurons in mice inoculated with street rabies virus by the intramuscular route.

Materials and methods

Three 28 days old ICR mice were inoculated with rabies virus obtained from the brain of a rabies infected dog. Each mouse was inoculated in their hind limb muscles with 0.03 ml of a 10^{-1} diluted aliquot equivalent to 10^{6} LD50. When the mice reached an advanced stage of the disease they were anesthetized by intraperitoneal injection of 1 ml of 30% chloral hydrate.

Then they were perfused by intracardiac route, initially with phosphate buffer (PB), pH 7.2, and then with a fixative solution composed of 2% glutaraldehyde and 4% paraformaldehyde prepared in PB, pH 7.2. Two mice that were not inoculated with the rabies virus (controls) were treated with the same procedure. This work was approved by the Ethics Committee of the Instituto Nacional de Salud (Bogotá, Colombia) according to Act No. 8 October 13 2011.

Brains were removed and 200 µm thick coronal slices were obtained using a vibratome. The brain slices were processed for electron microscopy using the following protocol: 1% osmium tetroxide postfixation (1 hour) followed by three PB washes and dehydration with ascending ethanol concentrations (50, 70, 80, 90, 95, 100%). Then the samples were treated with propylene oxide (PO), followed by infiltration by mixtures of OP and Epon-Araldite (EA) (2:1, 1:1, 1:2). The samples were embedded in pure resin (EA) overnight and then placed between two glass slides pretreated with a non-stick substance. These preparations were placed in an oven at 80°C during 24 hours for polymerization. Then the glass slides were separated using a razor blade to expose the embedded brain tissue. Cortex samples were extracted and joined with cyanoacrylate to previously polymerized resin blocks. This procedure allowed to maintain the orientation of the cerebral cortex and of the dendritic tree of pyramidal neurons. Semi-thin sections (500 nm) were obtained using an ultramicrotome and were then stained with toluidine blue for light microscope observation. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and observed with a Zeiss EM 109 electron microscope. Micrographs were taken using Kodak TMAX film and images were obtained by scanning the negative.

Results

In panoramic views (low-magnification) on the electron microscope, the distal dendrites of pyramidal neurons in the cerebral cortex of control mice were characterized by the presence of elongated and narrow mitochondria and by the diffuse pattern of microtubules filling the dendritic cytoplasm (Fig. 1A). At higher magnification, separated microtubules were observed within dendrites, arranged in parallel to the cell membrane (Fig. 2). Several ultrastructural alterations were found in distal dendrites of pyramidal neurons in the samples taken from

rabies-infected mice. There was loss of microtubules, while those preserved lost their parallel orientation and appeared disorganized. In addition, the long mitochondria disappeared (Figs 1B and 3 A-D). The cytoplasm of dendrites in samples from infected mice looks clearer because of the loss of mitochondria and microtubules.

The most noticeable finding was the numerous electron-dense and membranous structures within the distal dendrites of pyramidal neurons and in some axons (Figs 1B, 3, 4). These structures were not observed in the neuron soma nor in the apical dendrites. The electron-dense structures reached sizes ranging from 0.5 to 1.25 microns (n = 15) and were always located in the center of the dendrite branches separated from the cell membrane. The dendrites containing these structures were more thickened and showed a remarkable loss of mitochondria and microtubules. In addition, these electron-dense structures appeared to induce the formation of protuberances on the surface of axons (Fig. 3E). On the other hand, some vacuoles were observed within dendrites. These vacuoles seemed to totally or partially interrupt the continuity of the cytoplasm content and of microtubules (Fig. 5). Importantly, no virus particles were observed in the affected dendrites. Rabies virus particles and Negri bodies were observed only in the soma and apical dendrites of pyramidal neurons.

Discussion

The presence of narrow and elongated mitochondria is characteristic of distal dendrites of pyramidal neurons of the cerebral cortex (Peters, Palay & Webster, 1991). The absence of mitochondria and the marked loss of microtubules in the distal dendrites of cortical pyramidal cells in rabies-infected mice are very important features that partly explain the alterations in dendritic morphology, as previously reported by the Golgi technique (Torres-Fernández, Yepes & Gómez, 2007). There are numerous publications on the ultrastructure of nerve tissue affected by rabies virus in samples of humans (Iwasaki et al., 1985; De Brito, DeFantima & Tiriba, 1973; Sandhyamani et al., 1981; Manghani et al., 1986), animals (Matsumoto, 1963; Iwasaki & Clark, 1975; Fekadu, Chandler & Harrison, 1982; Charlton et al., 1987), and cell cultures (Hummeler, Koprowski & Wiktor, 1967; Hummeler & Atanasiu, 1996; Velandia et al., 2007). The vast majority of these studies refer to the intracellular localization of the rabies

virus, the formation of Negri bodies and, to a lesser extent, the general aspects of the ultrastructure of nerve tissue, especially the neuronal cytoplasm (perikaryon).

Few ultrastructural studies have been focused on dendritic arborization in samples processed as required for such analysis. We carried out this work to study the fine structure of the dendritic branching of pyramidal neurons of the cerebral cortex. This procedure facilitated the observation of ultrastructural features not previously reported in dendrites of nerve tissue infected with the rabies virus. We believe that the electron-dense structures formed within distal dendrites of rabies-infected tissue could correspond to some type of myelin-like figures derived from degenerating mitochondria. There are three reasons that support this hypothesis. 1. The distal dendrites of pyramidal neurons in normal tissue contain elongated mitochondria, while no mitochondria were observed in dendrites from rabies-infected tissue and in their place seemed be have been occupied by myelin-like figures. 2- According to scientific literature, myelin figures are formed from the transformation of membranous structures, mainly from mitochondria, in response to different conditions of cellular injury (Sjöestrand, Cedergren & Karlsson, 1964; Le Beux, Hetenyi & Phillips, 1969; Miguet-Alfonsi et al., 2002; Lin et al., 2012). 3- There are recent reports of mitochondrial dysfunction in rabies-infected cell cultures (Alandijani et al., 2013).

The myelin figures may also seem to be artifacts that affect mitochondria mostly by prolonged fixation in glutaraldehyde (Robards & Wilson, 1993). However, we processed the samples for electron microscopy few hours after the mice were sacrificed and fixed by perfusion. In addition, the myelin-like figures were not observed in controls. Moreover, the pathological origin of myelinated figures has been widely recognized in different tissue types (Le Beux, Hetenyi & Phillips, 1969; Miguet-Alfonsi et al., 2002; Castejon, 2008; Lin et al., 2012), and its mitochondrial origin has also been experimentally demonstrated (Le Beux, Hetenyi & Phillips, 1969; Schneeberger, Lynch & Geyer, 1976). However, the myelin-like figures we found were morphologically different from the typical images of concentric lamellae reported by other researchers (Le Beux, Hetenyi & Phillips, 1969; Castejon, 2008). The myelin figures previously observed in rabies showed the characteristic morphology of concentric lamellae (Matsumoto, 1963; Miyamoto & Matsumoto, 1967). They were found in

the perikaryon of cortical neurons. In contrast, the myelin-like figures described in this paper were more electron-dense and of irregular shapes, and were not found within the perikaryon.

Furthermore, our results differ from the ultrastructural findings in neuronal processes reported by other authors in nervous tissue infected with rabies. In a first study, mice were intracerebrally inoculated with a pathogenic variant (NC2) of the CVS fixed virus. Loss of organelles (mitochondria and endoplasmic reticulum) and partial destruction of neuronal processes were described. A decrease in the electron density of panoramic images was also observed (Li, Sarmento & Fu, 2005). A subsequent study on transgenic mice inoculated by intramuscular route with CVS fixed virus found swelling of mitochondria within the perikaryon and the proximal segment of apical dendrites of pyramidal neurons, but microtubules were preserved intact (Scott et al., 2008).

Our experiment was conducted under conditions closer to natural infection. We used intramuscular inoculation with street virus isolated from the brain of a dog infected with rabies. The vast majority of experimental rabies studies have used a laboratory virus (fixed virus). The differences in pathophysiology, including ultrastructural pathology, induced by the two types of virus (fixed virus vs street virus) are widely known (Miyamoto & Matsumoto, 1967; Tsiang, 1993; Hummeler & Atanasiu, 1996; Torres-Fernández et al., 2004; Torres-Fernández, Yepes & Gómez, 2007).

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Conflict of interest

The authors declare there are no conflicts of interest.

Author contributions

Orlando Torres-Fernández conceived and designed the experiments, performed animal experiments, imaged by electron microscope, analyzed the data, wrote the paper.

Jeison Monroy-Gómez performed animal experiments, processed the brain tissue for electron microscopy, imaged by electron microscope, analyzed the data, reviewed drafts of the paper.

Ladys Sarmiento processed the brain tissue for electron microscopy, imaged by electron microscope, analyzed the data, reviewed drafts of the paper.

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FIGURE 1.

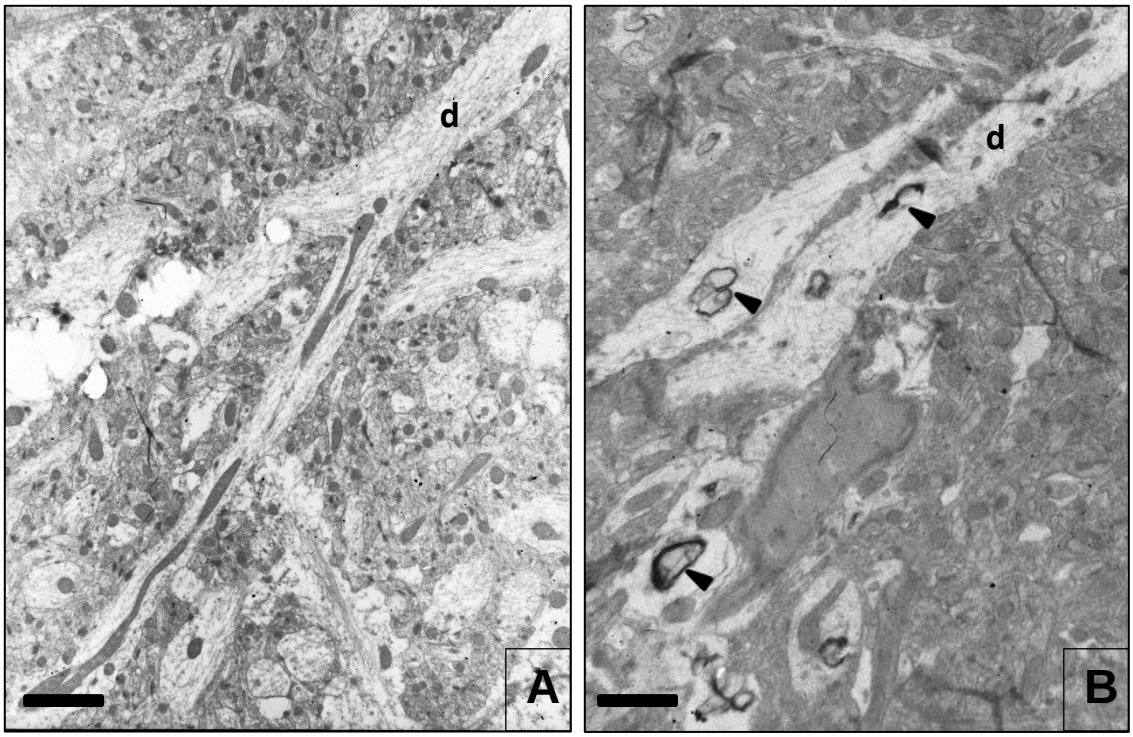


Figure 1. Panoramic (low magnification) electron microscope images of distal dendrites (**d**) from pyramidal neurons in the cerebral cortex of a control mouse (**A**) and in a rabies-infected mouse (**B**). Very long and narrow mitochondria and microtubules filling the cytoplasm are evident in the control mouse. Note some unusual structures with electron-dense border in the rabies-infected mouse (arrowheads). Scale bars: A = 2.2 µm; B = 1.6µm.

FIGURE 2.

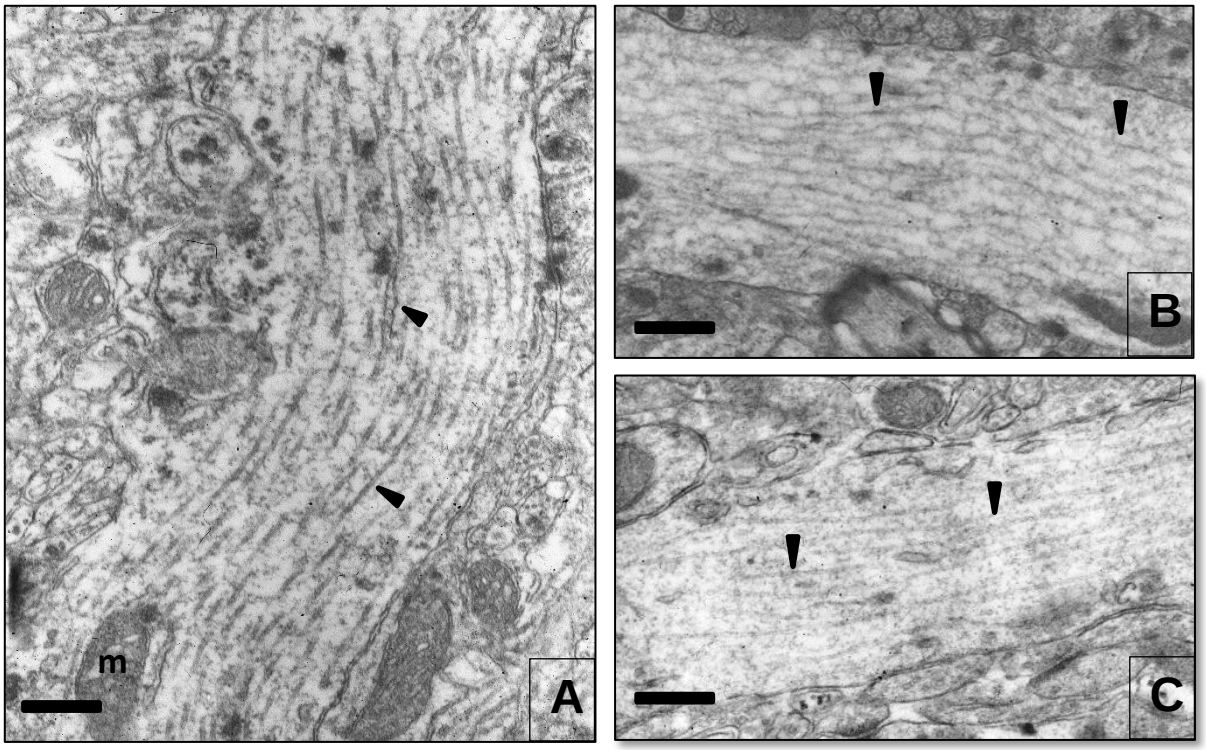
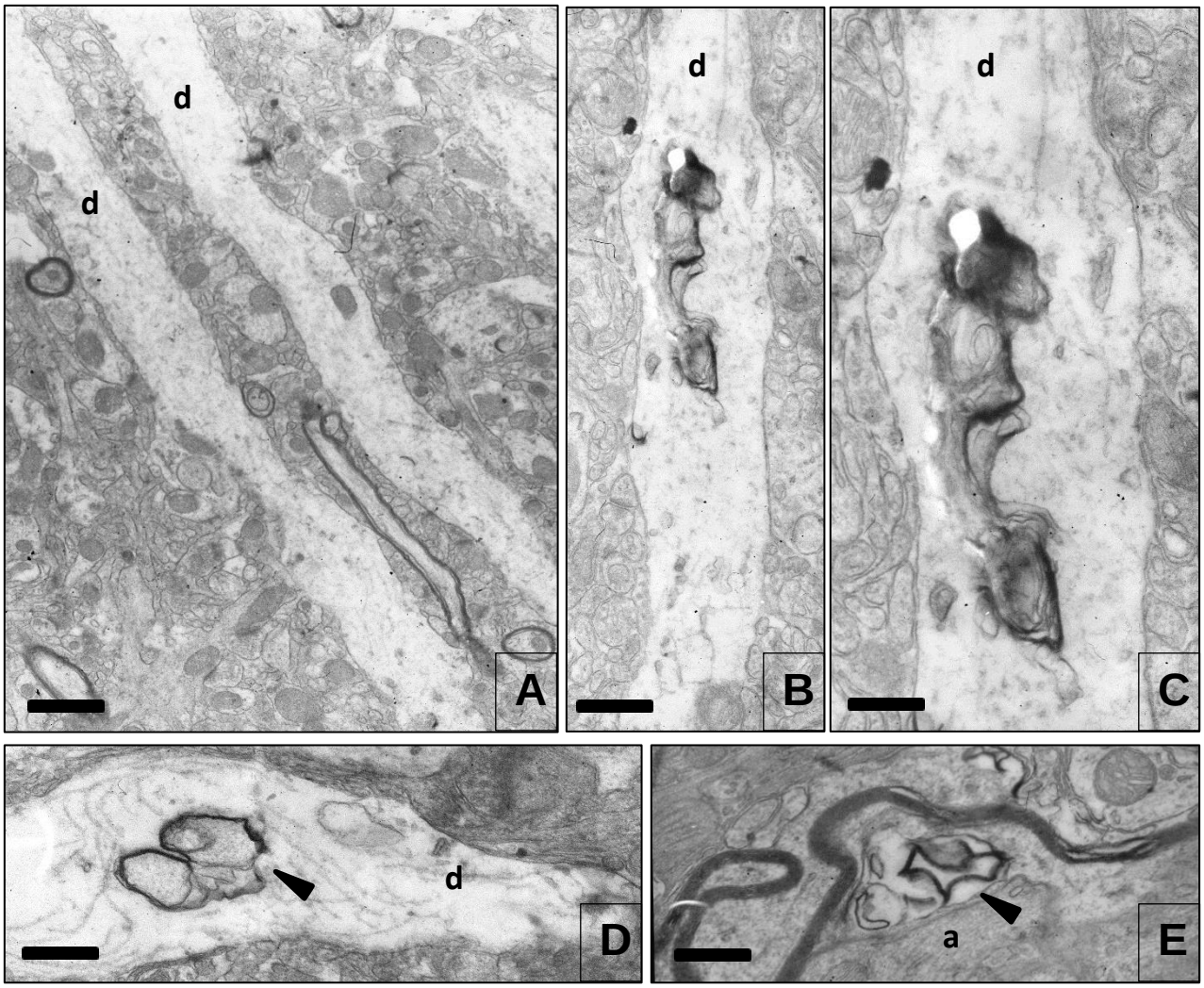


Figure 2. A - C. Three fragments of distal dendrites of pyramidal neurons in the cerebral cortex of control mice. Note the microtubules (arrowheads) maintaining a parallel orientation to the cell membrane. Mitochondrion (**m**). Scale bars: A = 0.39 µm; B = 0.71 µm; C = 0.39 µm.

337 FIGURE 3.



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340 **Figure 3. A - D.** Distal dendrites (**d**) of pyramidal neurons in the cerebral cortex of rabies-infected
341 mice. **A.** Panoramic image of two dendritic branches showing shortage of mitochondria and
342 microtubules. **B.** Dendrite containing an elongated myelin-like figure. Note the absence of
343 microtubules. **C.** Detail of image B. Note the electron-dense border and membranous areas. **D.**
344 Dendrite containing a myelin-like figure (arrowhead) surrounded by disorganized microtubules. **E.**
345 Fragment of an axon (a) displaying a varicosity containing a myelin-like figure (arrowhead). Scale
346 bars: A = 1.33 µm; B = 0.65 µm; C = 0.39 µm; D = 0.57 µm; E = 0.43 µm.

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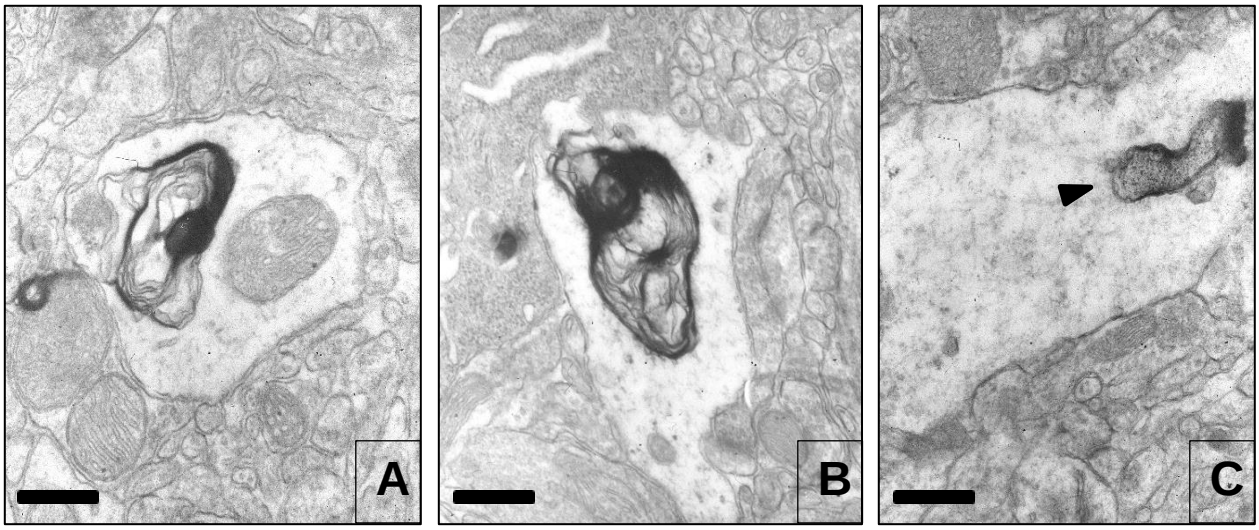
348 FIGURE 4.



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351 **Figure 4. A, B.** Sections of dendrites containing myelin-like figures in the cerebral cortex of
352 rabies-infected mice. Note how electron-dense areas blend with clearer membranous areas. **C.**
353 Fragment of a dendrite containing a mitochondrion (arrowhead) that is apparently suffering a
354 degenerative process. Scale bars: A = 0.38 µm; B = 0.54 µm; C = 0.59 µm.

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FIGURE 5.

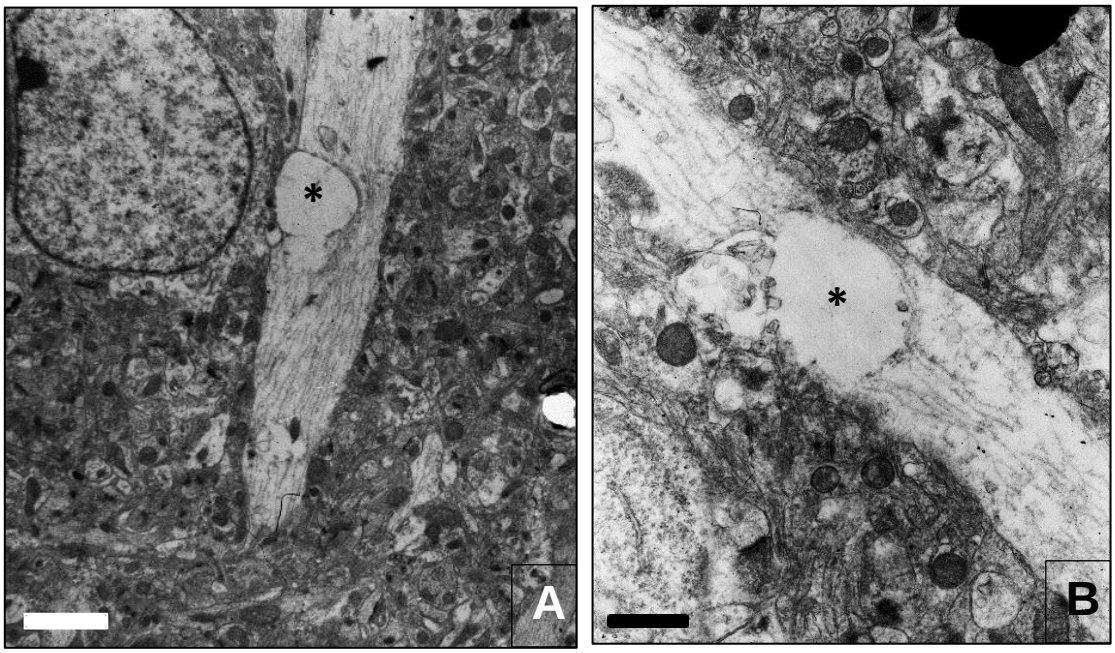

Figure 5. A, B. Distal dendrites of pyramidal neurons in the cerebral cortex of a mouse inoculated with the rabies virus. Note the vacuoles (*) interrupting the continuity of the microtubules and the dendritic cytoplasm. Scale bars: A = 2.2 µm; B = 0.71 µm.