Detection of macroglial cells in the fish optic nerve by intracellular injection in fixed tissue

A. Velasco, C. Lillo, D. Jimeno, E. Cid, E. Weruaga, J. Aijón and J.M. Lara

Dpto. de Biología Celular y Patología, Instituto de Neurociencias de Castilla y León, Universidad de Salamanca, 37007 Salamanca, Spain

SUMMARY

In this study we made intracellular injections of Lucifer Yellow fluorochrome into macroglial cells, astrocytes and oligodendrocytes of the fixed optic nerve of tench (*Tinca tinca*). From their three-dimensional morphology, we identified oligodendrocytes and at least four different types of astrocytes, both in the central zones of the nerve and in that forming part of the glia limitans. Moreover, we have identified and described groups of associated astrocytes.

Key Words : Optic nerve – Oligodendrocytes – Astrocytes – Lucifer yellow – Fish

INTRODUCTION

Neuroglial cells, both astrocytes and oligodendrocytes, play an important role in the correct functioning of the central nervous system (CNS). Astrocytes maintain extracellular ion concentrations, support neuronal and axonal migration during development, regulate neuronal survival, and control neuronal proliferation (review in Kettenmann and Ransom, 1995). Oligodendrocytes are highly specialized cells that form the myelin sheaths (Peters, 1964; Bunge, 1968). One oligodendrocyte can produce multiple myelin segments, enveloping up to 50 separate axons (Peters and Proskauer, 1969). However, there are few data on the role of oligodendrocytes during the development, start and control of myelinization, or their intervention in the process of myelin disappearance in certain brain diseases (Priestley, 1984; Grinspan et al., 1996).

In mammals, the neuroglial population could be one of the factors, among others, responsible for the difficulty of spontaneous regeneration of the CNS after a lesion. Astrocytes form a glial scar (Ramón y Cajal, 1928; Fedoroff et al., 1993) and could release several factors (Snider and Johnson, 1989) that impede axonal regeneration. Oligodendrocytes and myelin have inhibitor proteins that block the development of new axons. However, in several anamniote vertebrates, such as fish, that show continuous growth (Johns, 1977; Johns and Easter, 1977) and regeneration capacity (Sperry, 1948; Attardi and Sperry, 1963) of the visual system, neuroglial cells participate actively in these processes. Astrocytes do not form a glial scar after damage and oligodendrocytes have proteins that do not prevent regeneration of the axons (Jeserich et al., 1990; Bastmeyer et al., 1991).

Macroglial cells have been studied using different methods, such as the Golgi technique (Río Hortega, 1928), electron microscopy (Vaughn and Peters, 1967; Bunge, 1968) and immunohistochemical studies (Kettenmann and Ransom, 1995). Extensive knowledge about astrocytes is available thanks to the existence of specific antibodies against GFAP, vimentin, glutamine synthetase and S-100 (Dahl and Bignami, 1973; Cohen et al., 1993; Bignami, 1995; Donato, 1999). However, the study of oligodendrocytes has proved more difficult because their labelling with antibodies does not offer good results and most studies have been carried out in cell culture. In the last decade, the development of intracellular injection of different fluorochromes has allowed researchers to determine the three-dimensional morphology of astrocytes and oligodendrocytes in the optic nerve of mammals, both adults and during development (Butt and Ransom, 1989; Butt and Ransom, 1993; Butt et al., 1994a; Butt et al., 1994b; Weruaga-Prieto et al.,

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Correspondence to:
Dr. Juan M. Lara. Biología Celular, INCyL. Facultad de Medicina, Campus Unamuno, 37007 Salamanca, Spain.
Tel: +34-923294500 ext: 1856; Fax: +34-923294549. E-mail: rororo@usal.es

1996a; Weruaga-Prieto et al., 1996b). Knowledge of the three-dimensional morphology of neuroglial cells is fundamental if their function is to be comprehensively understood.

In the case of fish there are data about the peculiar immunological, histochemical and ultrastructural characteristics of macroglial cells in normal and regenerated optic nerves (Bunt, 1982; Maggs and Scholes, 1986; Lara et al., 1998; Levine, 1989; Maggs and Scholes, 1990). However, there is little information about the three-dimensional morphology of these cells and the interaction between them and optic nerve axons, or the possible morphological differences with mammalian neuroglia. In the present study, we employed intracellular injections of Lucifer Yellow to determine the three-dimensional morphology of the neuroglial cells of the normal optic nerve of fish. We also attempted to pinpoint the interrelationships between neuroglial cells and the axons of ganglion cells in the optic nerve of a teleost, the tench, that displays continuous growth and regenerative capacity of its visual system.

Material and Methods

All procedures used in this work were in accordance with the guidelines of the European Community Council Directive (86/609/EEC) and current Spanish Legislation (BOE 67/8509-12, 1988) for the use and care of animals.

We used 15 tench (*Tinca tinca* , L.) with a body weight between 100 and 150 g obtained from a commercial hatchery. The animals were deeply anaesthesized with 0.03% tricaine-methane sulfonate (MS-222, Sigma), rapidly decapitated, and the optic nerves were removed. Isolated optic nerves were fixed by immersion for 10 minutes with paraformaldehyde 4% in 0.1 M phosphate buffer (pH 7.4). Then, the optic nerves were washed overnight in saline phosphate buffer 0.1 M, pH 7.4 at 4° C.

In all cases the whole optic nerve was used. The special organization of the tench optic nerve as a folded ribbon (Fig. 1A) allowed us to unfold it and place it in an injection chamber with the help of entomology needles (Fig. 1B).

The chamber was fitted with a Leica DML FS microscope with special water-immersion ceramic objectives. Neuroglial cells were impaled with borosilicate glass microelectrodes containing an inner filament, which was prepared using a micropipette-puller (Sutter Instrument Co) by a NMN-21 micromanipulator (Narishige). Electrodes were back-filled with 5% (w/v) Lucifer Yellow (Sigma) in 0.1M Tris. They showed resistances ranging between 150 and 250 MΩ when filled with Lucifer Yellow. The fluorochrome was injected into the cells by a microiontophoresis system (WPI DCG-260), using a negative current

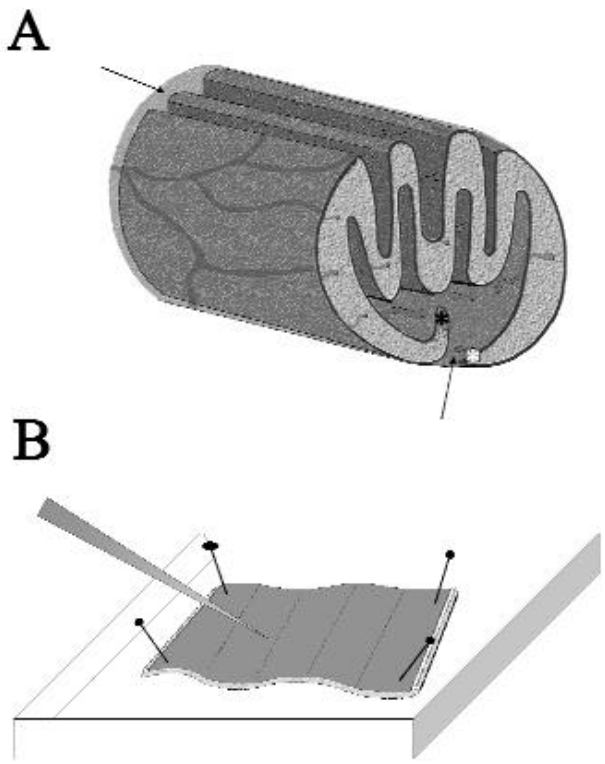

Fig. 1.- **A:** Organization of the tench optic nerve as a ribbon folder surrounded by conjunctive tissue (arrows). At the left extreme, young axons are seen (black asterisk). At the right extreme, mature axons are located (white asterisk). **B:** Unfolded optic nerve disposed in the injection chamber. Over the optic nerve the microelectrode with Lucifer Yellow used for the injection can be seen.

of 5 nA in 1 Hz pulses for approximately 500 ms. Neuroglial cells were impaled under manual control at the same time as Lucifer Yellow spread throughout the cytoplasm and their processes. The iontophoretic injections were maintained for 5 min. As the cells become stained, they were photographed with a camera (Leica DMLD) adapted to the microscope.

After injection of the neuroglial cells, the optic nerves were washed with 0.1 M phosphate buffer and postfixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4º C. Most nerves were processed for photo-oxidation of Lucifer Yellow to a 3,3`-diaminobenzidine tetrahydrochloride (DAB) precipitate, which allowed better analysis of their cytoarchitecture. In the process of photo-oxidation we followed the protocol described by Weruaga et al. (1996b). Briefly: optic nerves were incubated in 0.05 M Tris buffer (pH 7.4) with 0.02% of 3,3`-diaminobenzidine (DAB) for 1 h in the dark at 4ºC. Before this, the nerves were washed in a filtered solution with 0.04% DAB and 0.1% KCN in Tris buffer. The nerves were illuminated with blue light (100 W mercury lamp; x40 objetive) under a Leica DML FS microscope. The optic nerves were arranged on a slice, dehydrated and mounted with Entellan

(Merck). Prior to this, all the photo-converted cells were photographed.

For ultrastructural analysis after the photo-oxidation of the injected cells, the nerves were post-fixed with a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, 0.05% $CaCl_2$ in 0.1 M cacodylate buffer, 0.18 M sucrose (pH 7.4) overnight at 4ºC. The pieces were washed in 0.1 M cacodylate buffer, 0.18 M sucrose (pH 7.4) and post-fixed with 1% OsO_4 in distilled water containing 1% potassium ferricyanide for 2 h. Before dehydration, the pieces were subjected to block staining with 1% uranyl acetate in distilled water. Dehydration was performed using a graded series of cold acetone and Epon 812 (Taab) was employed as the embedding resin. Semithin sections (1µm thick) were stained with toluidine blue and ultrathin sections were mounted on formvar-coated one-hole grids, contrasted with uranyl acetate and lead citrate, and studied using a ZEISS EM-900 electron microscope.

Results

In most of the fixed optic nerves we obtained a high quantity of injected cells which were completely filled with the Lucifer Yellow. This permitted us to differentiate oligodendrocytes from astrocytes (Fig. 2A-C) and to analyze their cytoarchitecture.

The amount of injected oligodendrocytes was low. They had a small cellular body and numerous processes that ran parallel to the optic nerve axons (Fig. 2A,B). These cells had a round cellular body from which thin processes emerged, disposed in an oblique way that then changed directions in order to run parallel to the axons and constitute the myelin sheath (Figs. 2A,B).

In the case of astrocytes, we differentiated types on the basis of their disposition and their number of prolongations (Fig. 2C-G). We observed astrocytes with four different morphologies: 1) astrocytes with a stellate shape and a multipolar cellular body from which multiple long processes emerged. From the opposite extremities of the soma, two longer processes emerged that then bifurcated in their extremities to form a "T" (Fig. 2C). 2) Stellate astrocytes with multiple processes whose cellular body could not be distinguished. The prolongations started as a single straight ramification that usually bifurcated later (Fig. 2D). 3) Cells presenting their soma and most of their processes parallel to the axons (Fig. 2E). From the primary processes arose secondary prolongations, which were generally short and sometimes formed a "Y". 4) Astrocytes with their cellular body in the center of the fold and whose processes were directed mostly to the glia limitans. These displayed several thick ramifications from which other shorter ones emerged, directed mainly to the glia limitans (Fig. 2F). Moreover, in most astrocyte injections labeling was intermingled, such that some associated astrocytes were stained with Lucifer Yellow (Fig. 2G).

Some of the nerves in which fotoconversion was carried out were later processed for electron microscopy in order to determine ultrastructurally the type of glial cell labelled by means of intracellular injection and to establish its relationship with the rest of the elements of the optic nerve. We observed a DAB precipitate in the processes of some cells surrounding the axons as well as in the membrane of cellular bodies. In most cases, the labelled cellular body exhibited the typical ultrastructural morphology of astrocytes, with a clear nucleus and processes between the axons (Fig. 3A). Thick processes replete with DAB deposits seemed to correspond to astrocyte prolongations (Fig. 3B). Moreover, it was possible to observe some cellular bodies including axons, labelled with DAB, which had a darker nucleus (Fig. 3C). Occasionally myelin portions with DAB deposits were observed.

Discussion

We have demonstrated that intracellular injection of Lucifer Yellow in the fixed optic nerve of tench is an excellent technique for analyzing the neuroglial morphology and cytoarchitecture of astrocytes as well as those of oligodendrocytes. We have located and labelled complete oligodendrocytes, which have been impossible to characterize in fish using specific antibodies of oligodendrocytes as well as of the myelin sheath, such as O1, O4, Rip or GalC (Raff et al., 1978; Jeserich, 1983; Trotter and Schachner, 1989; Jeserich et al., 1990). The use of these antibodies produces excellent results *in vitro* and in very young animals (Bastmeyer et al., 1991; Jeserich and Stratmann, 1992), but *in vivo* the large quantity of myelin and the dense packing of axons has hitherto impeded detection of the cellular body and observation of the complete processes of these cells. Only one previous work has compared the morphology of oligodendrocytes in normal and regenerating goldfish nerves by means of intracellular injection (Ankerhold and Stuermer, 1999). The oligodendrocytes labelled by us are similar to those described in goldfish and resemble, although with fewer processes forming the myelin sheath, those described in the optic nerve of mammals (Butt and Ransom, 1989; Butt et al., 1994a; Weruaga-Prieto et al., 1996a). The data obtained from intracellular injections in the optic nerve of mammals as well as of fish suggest the existence of a single type of mature oligodendrocyte (Weruaga-Prieto et

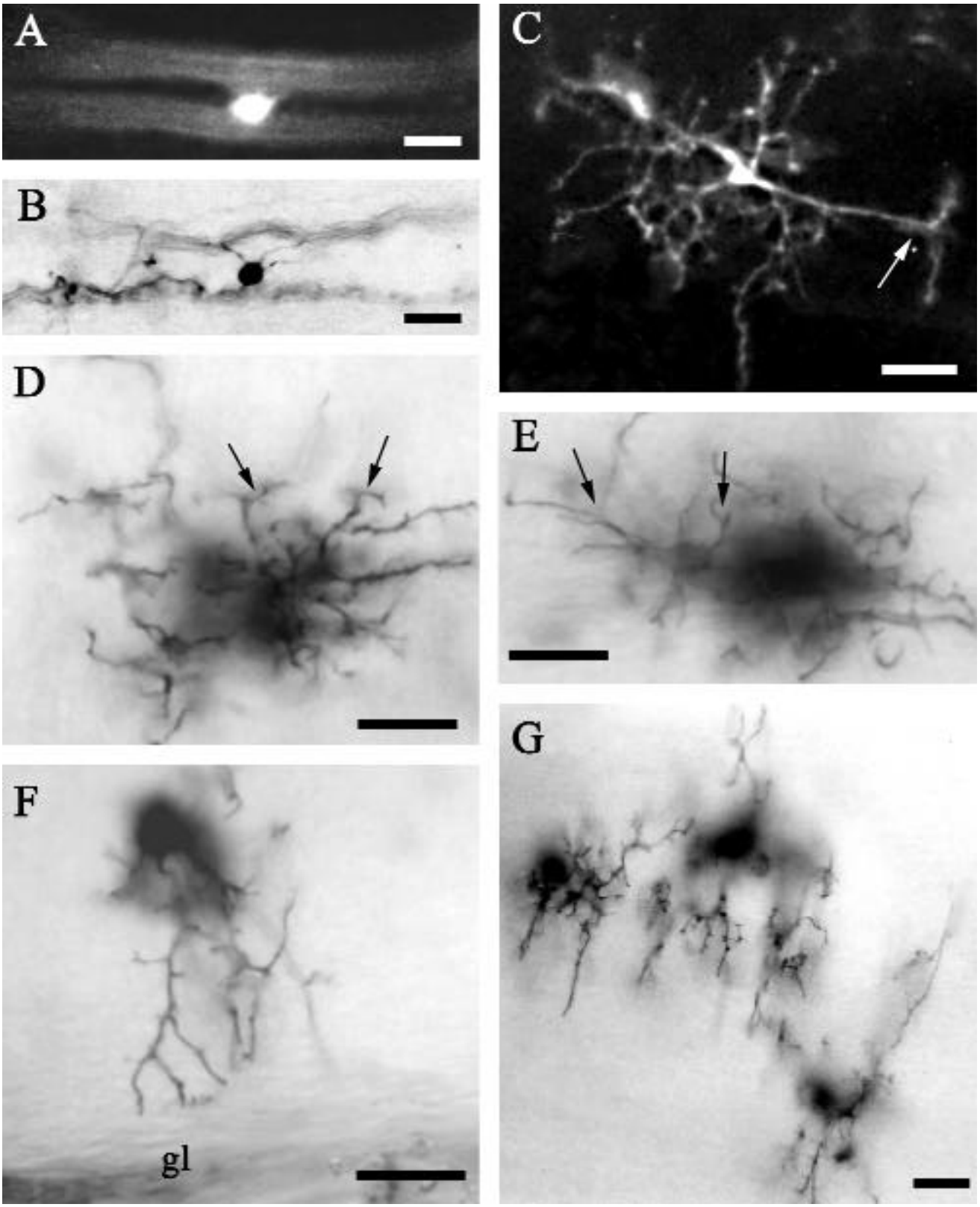

Fig. 2.- **A:** Typical oligodendrocyte with several processes disposed in "T" injected with Lucifer Yellow in the tench optic nerve. Scale bar: 50µm. **B:** The same oligodendrocyte after the photo-oxidation with DAB. The cellular body and processes can be seen in more detail. Scale bar: 50µm. **C:** Astrocyte with multiple processes injected with Lucifer Yellow. On the right of the cellular body, a long process with termination in "T" can be seen (arrow). Scale bar: 50µm. **(D-G):** Injected cells photoconverted with DAB. **D:** Starred astrocyte with multiple short thin processes, of which several are bifurcated in "T" (arrows). Scale bar: 50µm. **E:** Astrocyte disposed following the disposition of the optic nerve axons with numerous processes, most of them ramified in "Y" (arrow). Scale bar: 50µm. **F:** Astrocytes with their cellular body located in the centre of the fold and their processes disposed towards the glia limitans (gl). Scale bar: 25µm. **G:** Group of complete astrocytes labelled by diffusion of the fluorochrome when one of these was injected. Scale bar: 50µm.

al., 1996a). The oligodendrocytes visualized with intracellular injection seem to correspond to mature oligodendrocytes. However, recent studies carried out at our laboratory using electron microscopy (Lillo, 2001) have revealed that there are at least three different stages of oligodendrocytes in the adult optic nerve of fish, depending on the differentiation stage. This is apparently in agreement with the fact that the visual system of fish is subject to continuous growth. Some studies on the optic nerve of fish have suggested that the oligodendrocytes of these species are more similar to Schwann cells (Nona et al., 1992) than to mammal oligodendrocytes. With intracellular injections we failed to observe any oligodendrocytes similar to Schwann cells.

In the present work, we identified and differentiated several types of astrocytes. The only previous work on intracellular injections in the optic nerve of a teleost (Ankerhold and Stuermer, 1999) does not describe astrocyte morphology, and so far we have no news of any studies on the three-dimensional structure of astrocytes in the optic nerve of fish. The astrocytes of the optic nerve of fish, just like the oligodendrocytes, display some peculiarities with respect to those of mammals as regards both their morphology and their molecular composition. They differ from mammal astrocytes in that, at least to date, only one type of astrocytes has been described in the optic nerve of teleosts, called reticular astrocytes (Maggs and Scholes, 1990), whereas in mammals two types of astrocytes are considered (type 1 and type 2) (Raff et al., 1983; Miller et al., 1985). Additionally, in the cytoskeleton of the astrocytes of the fish optic nerve the typical proteins of this cellular type in mammals, such as GFAP, are not identified (Nona et al., 1989;Velasco, 1992), and they contain others which are characteristic of epithelial cells, such as cytokeratin (Giordano et al., 1989). Maggs and Scholes (1990) reported the reticular astrocytes of fish to be stellate cells, transversally organized, disposed at a right angle to the optic fibers and repeating themselves in regular intervals of 15 µm, forming a three-dimensional web. Moreover, these astrocytes contact with Ranvier nodules. Nevertheless, we have observed at least four different astrocyte morphologies, with a clear difference between those that take part in the formation of the glia limitans (Fig. 2F) and those located in the central zones of a fold of the nerve (Fig. 2 C,D,E). In addition, some studies performed with immunohistochemistry for S-100 (Velasco et al., 1997) and NADPH-diaphorase (Clemente, 1999) have also shown that the astrocytes disposed at the glia limitans display features such as a larger size and long processes, which clearly differentiate them from the rest of the astrocytes of the nerve, in agreement with the results of this paper.

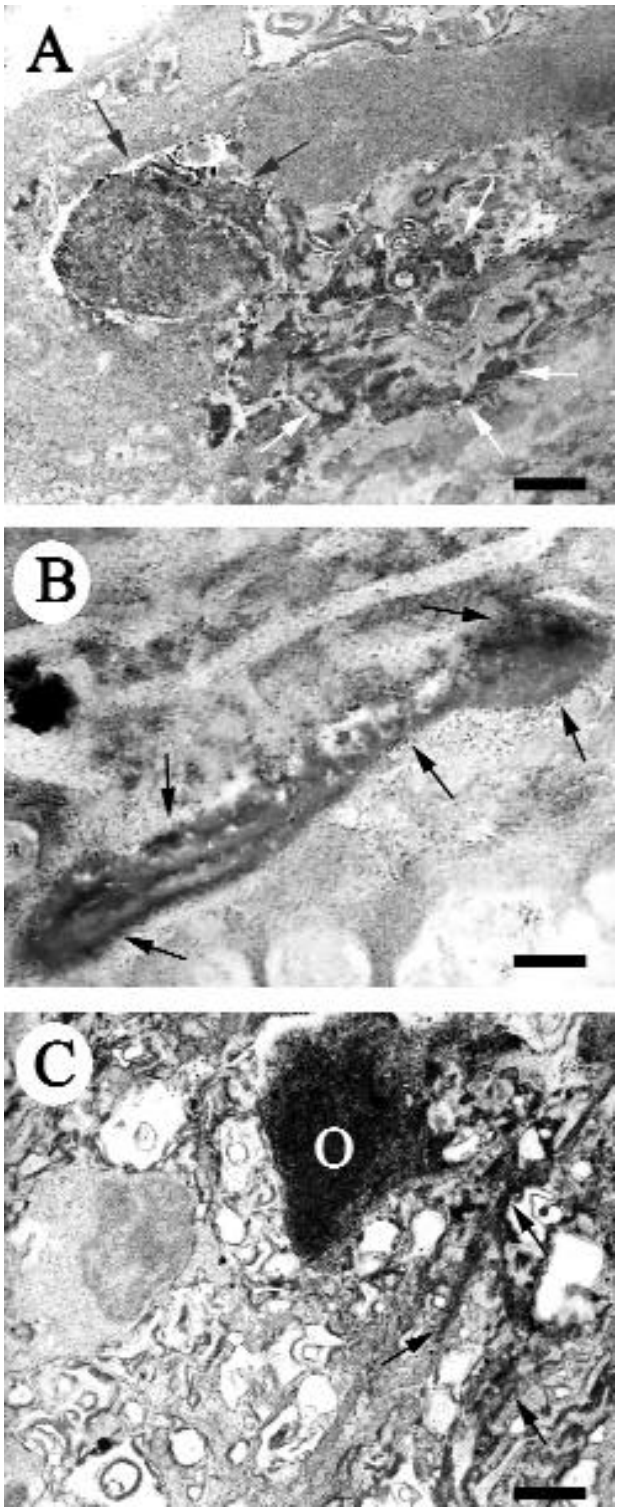

Fig. 3.- Electron microscopy images of the transverse section of the optic nerve where cells injected with Lucifer Yellow and the fluorochrome photooxidised with fluorescent light and DAB are observed. **A:** Cellular body of astrocyte (black arrows) and several processes of other cells with a precipitate of DAB in their membrane (white arrows). Scale bar: 15,84 µm. **B:** Cellular processes (arrows) of astrocyte completely full of DAB precipitate. Scale bar: 2616,9 nm. **C:** Labelled cellular body of an oligodendrocyte (O) with a dark nucleus and cytoplasm. Several myelin sheaths are also labelled (arrows). Scale bar: 15,84 µm.

Finally, we consider it of interest that in the present work we used a new technique that allows us to carry out injections or immunohistochemistry experiments with the nerve *in toto*, thanks to the unfolding of the optic nerve by means of its surgical manipulation after a short fixation.

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