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Dysmyelination, demyelination and reactive astrogliosis in the optic nerve of the *taiep* rat

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ABSTRACT

Taiep is an autosomal recessive mutant rat that shows a highly hypomyelinated central nervous system (CNS). Oligodendrocytes accumulate microtubules (MTs) in association with endoplasmic reticulum (ER) membranes forming MT-ER complexes. The microtubular defect in oligodendrocytes, the abnormal formation of CNS myelin and the astrocytic reaction were characterized by immunocytochemical and ultrastructural methods during the first year of life. Optic nerves of both control and *taiep* rats were processed by the immunoperoxidase method using antibodies against tubulin, myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP). *Taiep* oligodendrocytes are strongly immunoreactive against tubulin, indicative of a significant accumulation of microtubules. Early differentiated oligodendrocytes observed with electron microscopy show that MT-ER complexes are mainly present in the cell body. This defect increases during the first year of life; oligodendrocytes show large MT-ER complexes projected within oligodendrocyte processes. Using anti-MBP, there was a progressive reduction of immunolabeling in the myelin sheaths as *taiep* rats grew older. Ultrastructural analysis revealed severely dysmyelinated axons with a frequently collapsed periaxonal collar. However, through age the myelin sheath became gradually infiltrated by MTs, suggesting their contribution to premature loss of myelin in the *taiep* rat. Axons of one-year-old *taiep* rats were severely demyelinated. Modifications in astrocytes revealed by the GFAP antibody showed a strong hypertrophy with increased immunostaining in their processes. As demyelination of axons progressed, *taiep* rats developed a strong astrogliosis. The present findings suggest that in *taiep* rats the early abnormal myelination of axons affects the adequate maintenance of myelin, leading to a progressive loss of myelin components and severe astrogliosis, features that should be considered in the pathogenesis of dysmyelinating diseases.

Key words: myelin mutant, *taiep* rat, optic nerve, dysmyelination, demyelination, astrogliosis.

INTRODUCTION

Myelinogenesis is a complex process that requires a high degree of coordination in the expression of the myelin components. Myelin in the CNS is a highly specialized extension of the oligodendrocyte plasma membrane. In the rat, differentiated oligodendrocytes synthesize a large amount of myelin during the first postnatal month. Oligodendrocytes project several cellular processes that initiate the ensheathment of a target axon, thereby forming a multilamellar sheet of compact myelin. The sequence of events during myelination may be subjected to disturbances that affect the nervous system as a whole. Several diseases that involve the lack or loss of myelin have been identified (Hildebrand *et al.*, 1993). Mutations in specific genes of myelin structural proteins have been characterized (Sorg *et al.*, 1986; Campagnoni, 1988), nearly all of which result in severe dysmyelination (Nave, 1994). Inherited or acquired myelin disorders, with severe neurological damage, have a poor prognosis. Many myelin mutants have been described and intensively used to investigate normal and pathological aspects of myelination (Duncan *et al.*, 1987; Duncan, 1995; Nave, 1995; Lunn et. al., 1995; Griffiths, 1996).

The *taiep* rat is a myelin mutant in which an autosomal recessive mutation has been described (Holmgren *et al.*, 1989). However, the precise genetic defect has not yet been elucidated (Lunn *et al.*, 1995; 1997a). Homocygote *taiep* rats develop

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progressive neurological disorders, starting with a body tremor during the second postnatal week. Other clinical signs, such as ataxia, immobility episodes, audiogenic epilepsy and hind limb paralysis, progressively appear during the first year of life (Holmgren *et al.*, 1989). In the *taiep* mutant rat there is a severe deficit of CNS myelin formation resulting in continuous loss of myelin during the first year of life (Duncan *et al.*, 1992; Lunn *et al.*, 1997a); these alterations have not been evaluated in a detailed study over the same period.

Electrophysiological studies have shown that evoked responses in auditory, visual and somatosensorial systems are severely affected during early developmental stages of *taiep* rats (Benítez *et al.*, 1997; Roncagliolo *et al.*, 2000). The abnormal morphology, prolonged latencies, and reduced amplitudes of evoked responses have a good correlation with the dysmyelinating conditions observed in these rats.

The most conspicuous feature of *taiep* rat oligodendrocytes is the accumulation of microtubules (MTs) physically bound to the membrane profiles of the endoplasmic reticulum (ER). These structures will be referred to as MT-ER complexes in this paper. The formation of MT-ER complexes is well-established in *taiep* oligodendrocytes during the second postnatal week and has been related to the impairment in the intracellular transport of myelin components that occurs in these cells (Couve *et al.*, 1997). Furthermore, quantitative biochemical analyses of myelin proteins in the *taiep* mutant rat showed a reduced level in proteolipid protein (PLP), myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) (Möller *et al.*, 1997).

The purpose of this study was to characterize the progressive changes of MT-ER complexes in oligodendrocytes of *taiep* rats, myelin organization and astrocytes reaction, occurring during the first year of life. Immunocytochemical and ultrastructural studies were performed using the optic nerves of affected *taiep* rats as a model system of CNS tracts.

MATERIAL AND METHODS

Animals

Homozygous *taiep* rats and normal male Sprague-Dawley rats were obtained from the colony bred in the Faculty of Science, University of Valparaíso (Valparaíso-Chile). The animals were housed in plastic cages with food and water *ad libitum* under artificial dark-light cycle (12:12; lights on at 07:00 h) and room temperature of 23 ± 1°C. The immunocytochemical and electron microscopy studies were done using normal and *taiep* rats of 1, 6, and 12 months (n= 6 males in each group).

Sampling

The rats were anaesthetized with ether, decapitated, and both optic nerves were quickly dissected. The right nerve was fixed in Bouin fluid. The left nerve was divided in two halves; the prechiasmatic half was fixed by immersion in Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde, in 0.1 M cacodylate buffer, pH 7.4); the distal half was immersed in a triple aldehyde mixture (TAM) (2% paraformaldeyde, 2% glutaraldehyde, 1% acrolein, buffered to pH 7.4 with 0.1 phosphate) (Rodríguez, 1969). The aldehyde fixed halves were postfixed in 1% OsO4 and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. The Bouin fixed nerves were embedded in paraffin and used for immunocytochemistry.

Light microscopy immunocytochemistry

Serial longitudinal and transversal sections of the optic nerves (7-8 μm thick) were processed by the immunoperoxidase method (Sternberger *et al.*, 1970). Sections of optic nerves from control and *taiep* rats were simultaneously immunostained in the same staining session. Antibodies were used against the following antigens: 1) *Tubulin*. Rabbit policlonal anti-tubulin (kindly provided by E. M. Rodríguez, Valdivia,

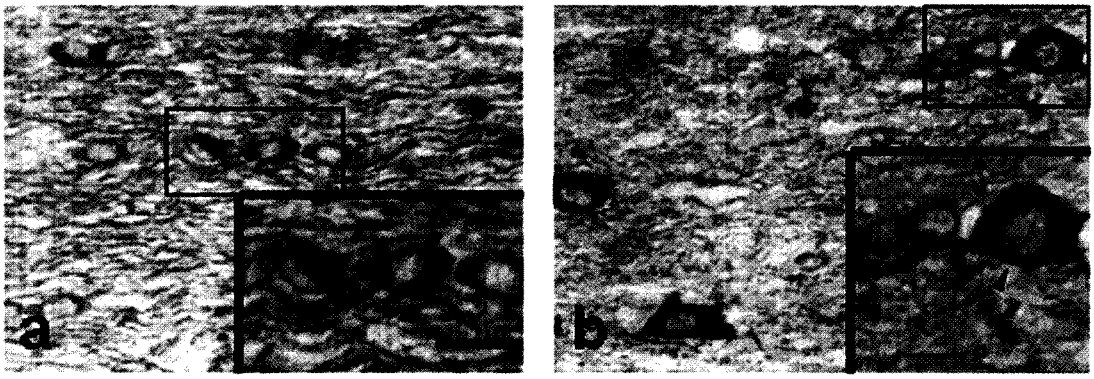

FIGURE 1

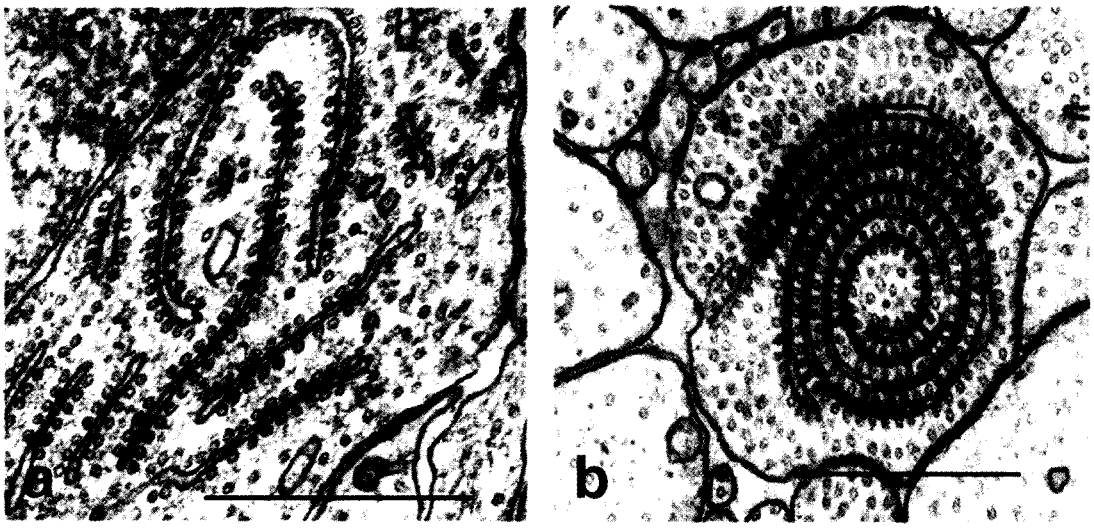

FIGURE 2

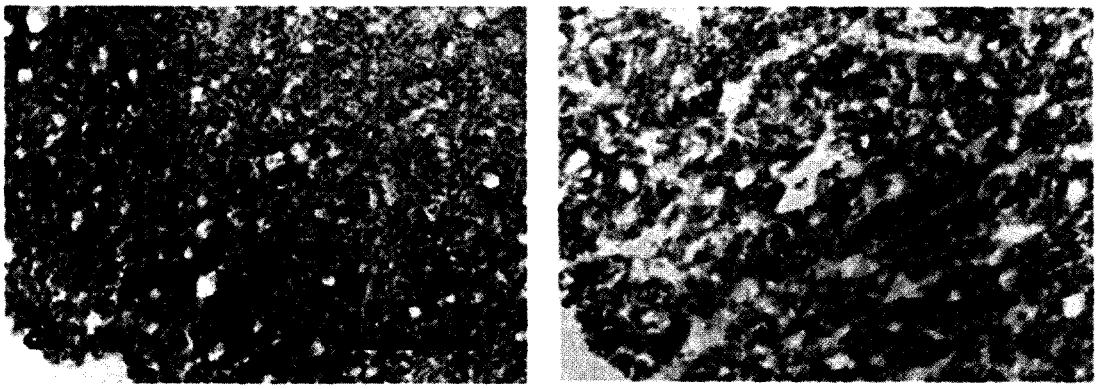

FIGURE 3

Figure 1 Light microscopy immunocytochemistry using anti-tubulin antibody. (a) In control rats (6 months old) low immunoreactivity of the oligodendrocytes can be observed; cells are comparatively smaller (Inset, Scale bar = 10 μm). (b) In *taiep* rats (6 months old) high immunoreactive cells are present; immunoreactivity is comparatively stronger (Insert, arrow)
Figure 2 Electron microscopy of MT-ER complexes in *taiep* oligodendrocytes. (a) Within the cell body of 1-month oligodendrocytes, accumulated microtubules appear closely associated with endoplasmic reticulum membranes. (b) In 1-year *taiep* rat, oligodendrocyte processes accumulate numerous microtubules. In the large process shown the membranes attached to microtubules reflect a spiral organization (Scale bars = 0.5 μm)
Figure 3 Light microscopy immunocytochemistry using MBP antibody. (a) Cross section from optic nerve of control animals (12 months old) with high reactivity in myelin. (b) Cross section from optic nerve of *taiep* rat (12 months old) treated with MBP antibody. The fascicular area shown is in equal magnification with the control; a low reactivity against the MBP antibody in myelin is observed. (Scale bar = 50μm)

Chile) (Rodríguez *et al.*, 1985) for the specific marking of microtubules was used at a dilution of 1:5000. 2) *Myelin basic protein* (MBP). Rabbit policlonal anti-Myelin Basic Protein (MBP) (Zymed Laboratories Inc., San Francisco, CA) was used at a dilution of 1:250. 3) *Glial fibrillary acidic protein*. Monoclonal anti-Glial Fibrillary Acidic Protein (GFAP) (mouse IgG1 isotype) (Sigma-Biosciences, St. Louis, MO), a specific marker for astroglia, was used at a dilution of 1:500.

Incubation in the primary antibody was done in a moist chamber for 18 h at room temperature. The secondary antibody was used at a dilution of 1:50. The PAP complex (provided by E.M. Rodriguez, Valdivia, Chile) was used at a dilution of 1:75. All antibodies were diluted in TRIS buffer at pH 7.8, containing 0.7% non-gelling seaweed gelatin, lambda carrageenan (Sigma) and 0.5% Triton X-100 (Sigma). The sections were visualized by the diaminobenzidine reaction.

Ultrastructural immunocytochemistry

The tissue was fixed in a mixture containing 2% paraformaldehyde, 0.5% glutaraldehyde, and 15% saturated picric acid solution buffered to pH 7.4 with 0.1 M monosodium-disodium buffer. After washing in the same buffer for 30 min, tissue blocks were fixed in 0.25% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for two h. The tissue was then washed in distilled water for 15 min, dehydrated and embedded in butyl-methyl-methacrylate (Rodríguez *et al.*, 1984). Ultra-thin sections mounted on nickel grids were treated with 1% H_2O_2 for 10 min and washed in distilled water. This was followed by a few rinses in TRIS buffer, pH 7.8. For immunostaining, the immunoperoxidase-silver methanamine method (Rodriguez *et al.*, 1994) was used. Anti-GFAP (Sigma) was used as primary antibody at a dilution of 1:500. Controls included (1) immunostaining omitting incubation in the primary antibody, and (2) silver methenamine staining without previous immunoperoxidase staining.

RESULTS

1. Oligodendrocytes

Immunocytochemistry

Oligodendrocytes were labeled by anti-tubulin. This antibody is specific for microtubules, the main cytoskeletal component of oligodendrocytes. In both the *taiep* and control rats, the immunoreactive oligodendrocytes were arranged in rows, parallel to the axons (Figs. 1 a-b). The primary difference between both groups of rats refers to the size of oligodendrocytes cell bodies and the intensity of their immunoreactivity. *Taiep* oligodendrocytes are strongly immunoreactive to anti-tubulin, indicating a significant accumulation of tubulin in their cytoplasm and their cell processes.

Electron Microscopy

Ultrastructural analysis of early-differentiated oligodendrocytes reveals microtubules in association to membranes profiles that are connected to the endoplasmic reticulum network. These MT-ER complexes are mainly accumulated in the cell body of one-month-old *taiep* oligodendrocytes (Fig 2a). As mutant rats age, these complexes progressively enlarge, accumulate, and project within the cellular processes. At one year of life, oligodendrocyte cell bodies show a high accumulation of microtubules projected into the cell processes in close contact with an enlarged endomembranous network composed of cisterna and tubules that are directly associated with the endoplasmic reticulum (Fig 2b).

2. Dysmyelination and Demyelination of Axons

Immunocytochemistry

Myelin was labeled using anti MBP. This antibody is specific for one of the major myelin proteins. The optic nerves of mature *taiep* rats (6 months) showed a decreased

amount of immunoreactive MBP as compared to young *taiep* rats (1 month). Furthermore, one-year-old *taiep* rats displayed a reduced MBP immunoreactivity as compared with control rats of the same age (Fig 3 a-b).

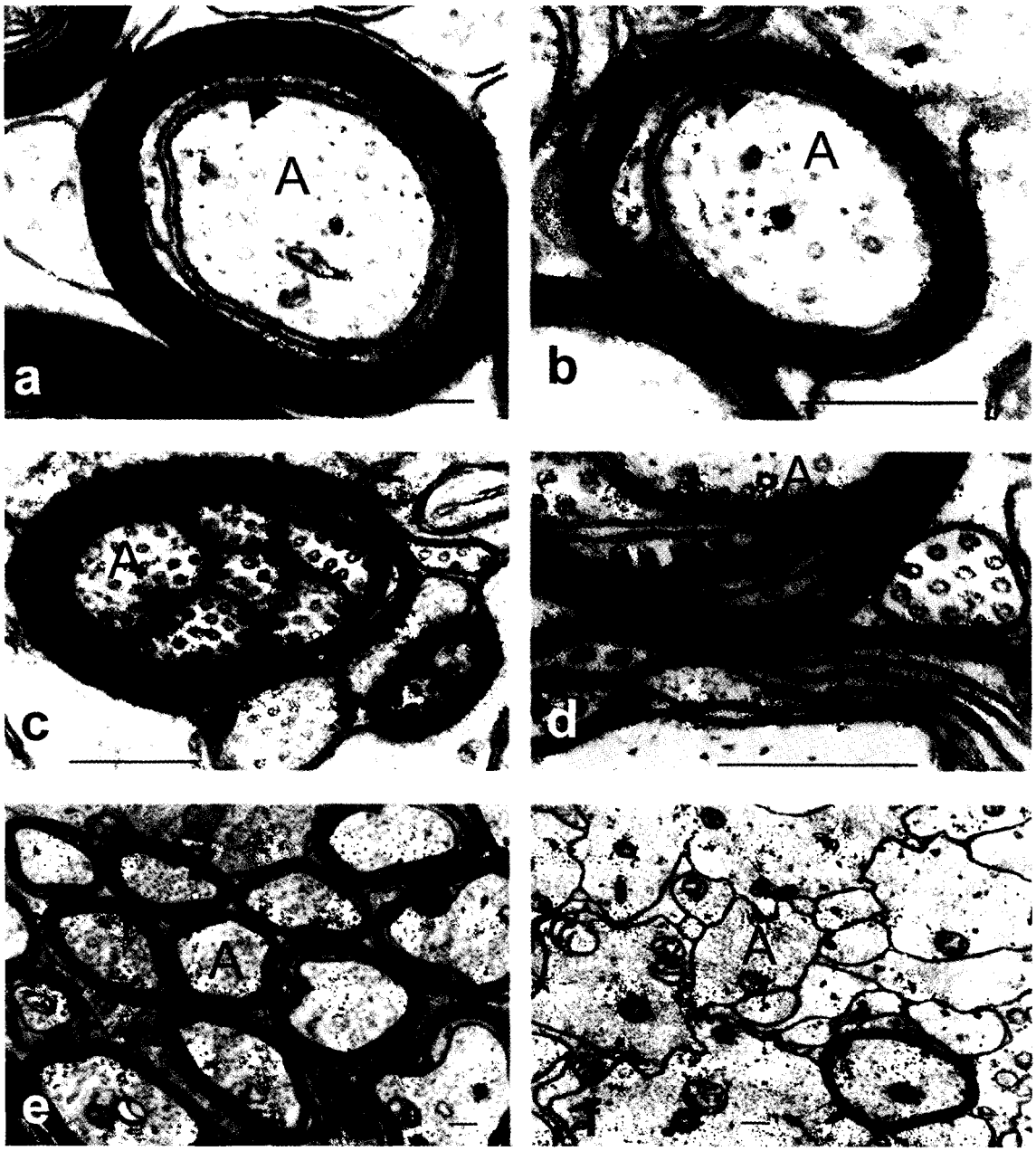

FIGURE 4

Figure 4 Electron Microscopy of myelinated axons in one-, six- and twelve-month-old rats (a-f). At one month: (a) Cross section of myelinated axon from the optic nerve of control animal. The normal tight organization of the myelin sheath is evident and the periaxonal collar (arrow) surrounding the periaxonal space is clearly arranged. (b) Cross section of myelinated axon from optic nerve of *taiep* rat. There is an abnormal organization of the myelin sheath, with a collapsed periaxonal collar (arrow) and a slightly compact arrangement. At six months (c-d) Cross sections of *taiep* myelinated axons show a high infiltration of myelin with microtubules. Decompacted myelin profiles become evident in both figures. Small oligodendrocyte microprocesses filled with microtubules are present (arrow). At 12 months (e) Cross section of normally myelinated axons in control rats. (f) In *taiep* rats most of axons are demyelinated. (Axons, A; all scale bars = 0.25 μm).

Electron Microscopy

Myelinated axons from *taiep* and normal rats were analyzed. The ultrastructural analysis showed that in *taiep* rats, the myelin sheaths are thinner; no more than three or four interperiod lines form the myelin sheaths. *Taiep* myelinated axons are clearly hypomyelinated when compared with control rats (Fig 4 a-b). In one-month *taiep* optic nerves, approximately 50% of the myelinated axons showed a total loss of the oligodendrocyte cytoplasmic periaxonal collar (Fig 4b). Abnormal compaction of myelin sheath was another relevant feature that characterized *taiep* myelin in CNS (Fig 4b). At six months, myelinated axons of *taiep* rats showed a high infiltration of myelin with microtubules (Fig 4 c-d). Decompacted myelin profiles were frequently observed at this age in *taiep*, and small oligodendrocyte microprocesses filled with microtubules were also present (Fig 4d). In one-year-old *taiep* rats, there was a severe demyelination of axons as a result of a progressive myelin loss in comparison with control rats (Fig 4 e-f).

3. Astroglial cells

Immunocytochemistry and Electron Microscopy

Astrocytes were visualized using the anti-GFAP antibody. By comparing transversal (Fig 5 a-b) and longitudinal (Fig 5c) sections, it becomes clear that processes of astrocytes preferentially orientate parallel to the axons. The nerve of *taiep* rats displayed a higher density of both astrocytes and astroglial processes at one year, as compared with age-matched control animals (Fig 5 a-b). At the conventional ultrastructural level, hypertrophied cells, which most likely correspond to astrocytes, showed a high density of glial filaments (Fig 5 d). The astroglial nature of these cells was confirmed by applying ultrastructural immunocytochemistry. Thus, the filaments of these cells were strongly immunoreactive with the anti-GFAP antibody (Fig 5e). Ultrastructural immunocytochemistry further revealed that the GFAP-immunoreactive filaments became organized into different bundles that partially filled the astroglial process (Fig 5f). These bundles would correspond to the GFAP immunoreactive fibres seen in astrocytes under the light microscope.

DISCUSSION

The *taiep* mutant represents an interesting model to study the pathogenesis of dysmyelinating diseases in the CNS. In this study we provide new evidence for the understanding of this pathology through the use of immunocytochemical and electron microscopy analysis. The accumulated microtubules observed in the cytoplasm of oligodendrocytes are always associated with endoplasmic reticulum membranes forming MT-ER complexes. During the first stages of myelination these MT-ER complexes are restricted to the cell body. As rats grow older, MT-ER complexes form an enlarged network of microtubules and membrane profiles projected into the oligodendrocyte processes. Microtubules play a key role in the normal intracellular transport process of lipids and proteins into specific myelin domains of oligodendrocytes (Dyer and Benjamins, 1989; Wilson and Brophy, 1989). The increase of immunoreactive tubulin observed in oligodendrocytes of *taiep* rats has a good correlation with the ultrastructural observations. This defect defines *taiep* mutation as an accumulative alteration with blockage in the normal intracellular progress of myelin components.

This study also characterizes myelin organization in dysmyelinated axons, describing the effect of MTs accumulation on the demyelination process. It is now well accepted that the early formation and maintenance of myelin sheaths require the participation of cytoskeletal elements (Kalwy and Smith, 1994). The transport of mRNA towards glial processes has been demonstrated experimentally (Ainger *et al.*, 1993; Brophy *et al.*, 1993). Myelin basic protein (MBP), the major peripheral membrane protein of CNS myelin, is

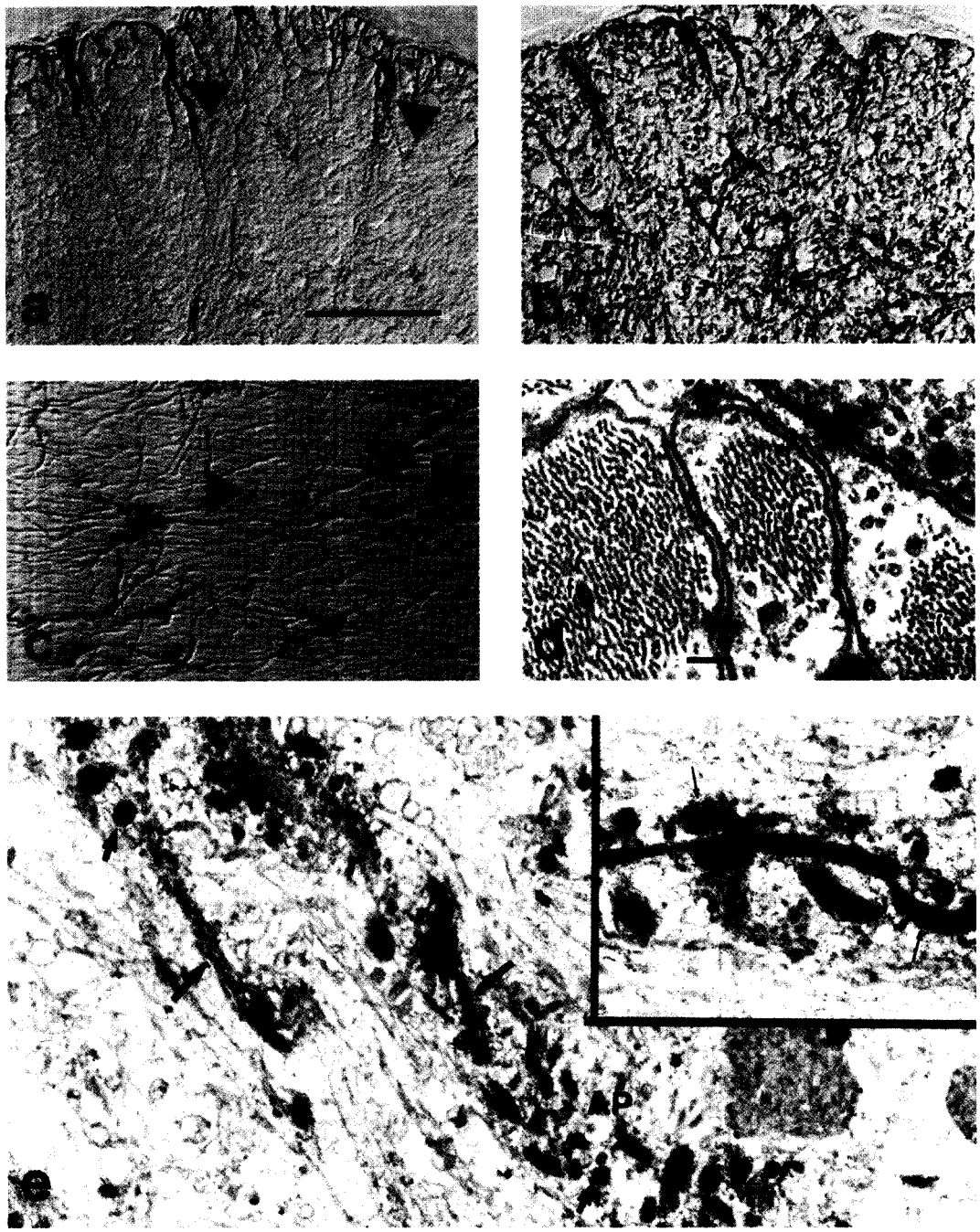

FIGURE 5

Figures 5 Light microscopy immunocytochemistry using GFAP antibody (a-c). (a) Cross section from optic nerve of control animals (12 months old). A low immunoreactivity of astrocytic cells is observed (arrows, scale bar = 50μm). (b) Cross section from optic nerve of *taiep* rat (12 month old). The fascicular areas are comparative with the controls (same magnifications) and show strong reactivity against the GFAP antibody. (c) Longitudinal section from optic nerve of *taiep* rat (12 months old) treated with GFAP antibody. Highly immunoreactive astrocytes are clearly observed with strong reactive processes against the GFAP antibody (arrowheads). Electron Microscopy (d-e). (d) Cross section of the optic nerve from 12-month-old *taiep* animals. Astrocytic processes containing increased amount of glial filaments (filaments, f) can be observed. (Scale bar = 0.1μm). (e) Immunoperoxidase-silver methenamine staining using anti-GFAP. In ultrathin methacrylate section of *taiep* optic nerve, astroglial processes (AP) appear partially filled with immunoreactive bundles of filaments transversally (short arrows) and longitudinally (long arrows) cut. A detailed magnification of an astroglial process showing the silver grains on bundles of filaments (Inset, arrows).

encoded by mRNAs that are translocated to the myelinating processes of oligodendrocytes to produce the normal assembly of myelin. The specific mechanism of MBP-mRNA transport and the correct delivery of MBP to myelin subdomains are specific features of oligodendrocytes (Brophy *et al.*, 1993). Microtubular components have been proposed as an important factor for the spatial segregation of myelin protein mRNAs within the oligodendrocytes processes (Colman *et al.*, 1982; Trapp *et al.*, 1987). The reduced MBP immunoreactivity in the CNS of the *taiep* rat, together with the reduced thickness of the myelin sheets, as was described here, could reflect the alteration in the translocation of MBP to the domain where this myelin protein is maintained. Microtubules are essential for normal myelination process Their organization, stability, and polarity orientation in the oligodendrocyte reflect the complex functions of this cell (Lunn *et al.*, 1997b), and any defect that disrupts this process affects the entire myelination process.

Taiep mutation causes severe hypomyelination of the CNS with dysmyelinated axons and their progressive demyelination during the first year of life. Dysmyelination in the central nervous system of the *taiep* rat could be characterized by a poor myelin formation and a progressive loss of myelin from the *taiep* CNS as the animals increase in age (Lunn *et al.*, 1997a). In this study we show that in *taiep* myelinated axons the periaxonal collar appear collapsed, suggesting the abnormal formation and maintenance of myelin (see Fig 4b). Intense MAG immunolabeling has been observed over oligodendrocyte cell bodies and processes (LT O'Connor *et al.*, personal communication), indicating a failure in the translocation process of myelin components. Accumulated MTs disrupt the intracellular adhesion of compacted membranes in *taiep* myelin, producing a decompaction of the myelin sheet and the formation of several oligodendrocyte microprocesses filled with microtubules.

The severe loss of myelin progresses with a strong reactive astrogliosis over the first year of the *taiep* mutant's life, a reaction that has also been observed under *in vitro* conditions (BA León *et al.*, personal communication). In many myelin disorders astrocytes respond with a reactive astrogliosis (Eddleston and Mucke, 1993), a cellular reaction that is mainly characterized by the hypertrophy of astrocytes and the increase in GFAP immunostaining (Mucke and Eddleston, 1995). The enhanced expression of intermediate filaments of astrocytes is a general reaction to damage in the CNS. Reactive astrogliosis occurs in CNS demyelination, as in multiple sclerosis, the most common demyelinating disease in humans. In *taiep* rats there is a severe astrocytic reaction characterized by hypertrophied astrocytes whose processes contained a large number of GFAP-immunoreactive bundles of filaments. Modifications of astroglia associated with genetic dysmyelination may vary in different myelin mutants. The *jimpy* mouse develops a strong gliosis in CNS, while other mutants like the *shiverer* and the *quaking* mouse exhibit a mild gliosis (Jacque *et al.*, 1986; Duncan, 1995). Astrocytes may participate in the removal of myelin from affected areas of CNS as astrocytic cytoplasmic processes fill the space resulting from the loss of myelin. However, although astrogliosis contributes to the healing of CNS injuries, pathological effects that prevent remyelination can also take place (Eng and Lee, 1995).

In summary, the present findings in *taiep* lead us to suggest that the alterations in the myelinated axons could be explained by a primary defect characterized by conspicuous MT-ER complexes in oligodendrocytes that triggers a dysmyelination followed by a progressive demyelination of CNS axons. These changes carried out a strong gliotic reaction that could explain the large loss of myelin in one-year mutant rats.

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