

Ultra structural evidence of axonal regeneration following intracranial transection of optic nerve

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Abstract

OBJECTIVE: The present work was aimed at studying the ultra structural changes of the proximal (retinal) stump of the intracranially transected optic nerve of the rat for any possible regenerative ability.

METHODS: Specimens were collected one (1 wpo) and four weeks(4 wpo) after the transection and the cross sections of the stumps were studied by electron microscopy by dividing them into three zones, (1) the central zone, (2) the intermediate zone, and (3) the peripheral zone.

RESULTS: The present results showed evident morphological changes in these zones both in the 1 wpo and 4 wpo groups. The signs of degeneration were more marked in the central zone than in the peripheral zone and they were more prominent in the 1 wpo group than in the 4 wpo group. The most prominent sign of the degeneration was loss or lack of the healthy myelinated axons. The main evidence of the regenerative ability was the reappearance of the apparently healthy myelinated axonal profiles, with a parallel decrease of the non myelinated ones. This regenerative feature was more prominent peripherally and might be an indication that ischemia was the cause of optic nerve degeneration.

CONCLUSION: The present work revealed a clear morphological evidence of the regenerative capability of the intracranially transected optic nerve though it is considered as a part of the CNS.

INTRODUCTION

Most neurons in the mammalian brain are generated embryonically during the restricted phases and the mature mammalian brain is characterized by a relatively constant number of neurons. There is still a low rate of constitutive gliogenesis and a more restricted neurogenesis in the adult mammalian central nervous system (CNS) (Rakic

2002). It has long been thought that neurons generally are not replaced in the mammalian brain after injury or during the course of a disease. Interestingly, several recent studies have reported compensatory proliferation and neurogenesis in response to injury or disease in the mammalian brain (Emsley *et al.* 2005). Axon growth is a highly

regulated process that requires stimulating signals from extracellular factors. Growth factors, especially neurotrophins that act via receptor tyrosine kinases have been heavily studied as extracellular factors that stimulate axon growth (Zhou & Sinder 2006). Light and electron microscopic studies of the intracranial optic nerve transection suggest that injuries to the optic nerve in the cranial cavity and the orbit result in microscopic changes, not only in the optic nerve but also on the retina. Interruption of the intra-orbital part of the optic nerve of adult rats is normally followed by extensive cell death in the retina. About 90% axotomised retinal ganglion cells (RGCs) disappear within one month of injury (Alleut *et al.* 1984; Villegasperef *et al.* 1988; 1993). There is considerable degeneration of the proximal (retinal) stump, perhaps because of damage to the vasculature (Grafstein & Ingoglia 1982; Richardson *et al.* 1982; Giftchristor & David 1988). The axotomised RGCs re-express growth associated protein (GAP-43) (Doster *et al.* 1991; Zeng *et al.* 1992a) and in the first few days after injury produce a large number of axonal sprouts close to the cut retinal stump of the nerve (Hall & Berry 1989; Zeng *et al.* 1991). Taylor *et al.* (2009), has suggested that CNS axons synthesize proteins locally, maintaining a degree of autonomy from the cell body. You *et al.* (2000), have shown a critical relationship between the distance of axotomy on optic nerve and the regenerating retinal ganglionic cells in adult hamsters. Axonal regeneration can occur within hours of injury, the first step being the formation of a

new growth cone. For sensory and retinal axons, regenerative ability *in vivo* correlates with the potential to form a new growth cone after axotomy *in vitro* (Verma *et al.* 2005). Verma *et al.* (2005) in their study have shown that the ability to regenerate a new growth cone depends on local protein synthesis and degradation within the axon. Following an intracranial lesion of the optic nerve, a much higher proportion of the injured RGCs survive (Madison *et al.* 1984; Misantone *et al.* 1984), but RGCs fail to up regulate GAP-43 after injury to this part of the optic nerve (Doster *et al.* 1991). Recently, it was shown that the basic helix-loop-helix transcription factor oligodendrocytes factor 2 (Olig2) is greatly up-regulated in endogenous precursors after injury (Buffo *et al.* 2005). Interestingly, repression of Olig2 in proliferating precursors leads to the production of immature neurons indicating that Olig2 acts as a suppressive non-neurogenic signal *in vivo* and thus one of the first non-permissiveness signals that has been found to act *in vivo* in the mammalian brain (Buffo *et al.* 2005). Stone *et al.* (2010) in their recent study has suggested that axonal regeneration might occur from dendrite. According to them regulation of microtubule dynamics and polarity is key to initiating regeneration of an axon from a dendrite. The present work aims to reinvestigate the apparently unlogic results reported by Zeng *et al.* (1992b) according to which intracranial transection of the optic nerve though associated with survival of greater number of RGC but have shown a lesser regenerative response.

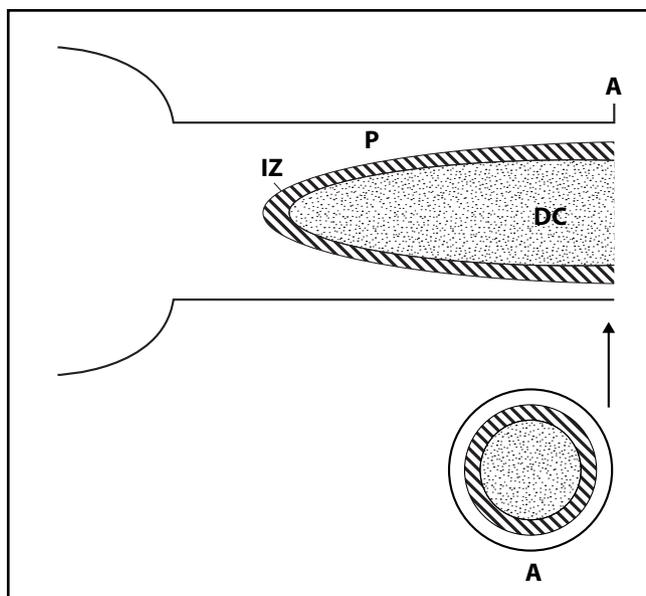


Fig. 1. A diagram illustrating the degenerative core, the central zone (DC, stippled), intermediate zone (IZ, hatched), peripheral zone (P) of the proximal stump of the optic nerve. A is the site of transection on longitudinal (above) and TS below (circle) which lies about 7 mm behind the eyeball intracranially (modified from Zeng *et al.* 1992b).

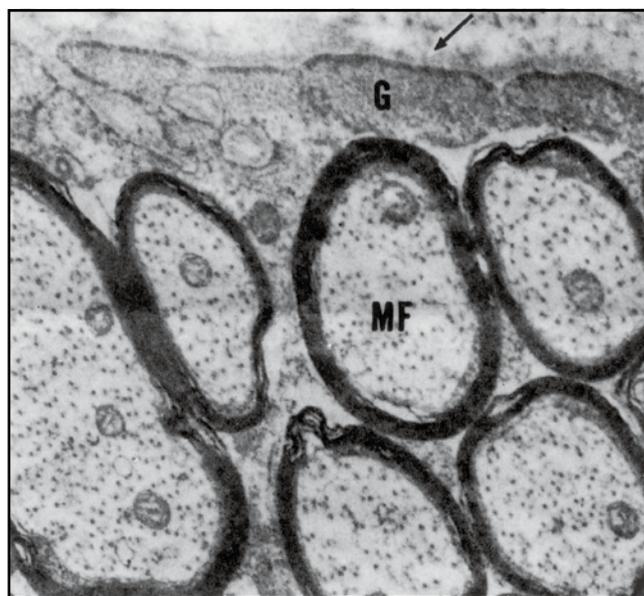


Fig. 2. Electron photomicrograph of retro-orbital healthy control optic nerve showing group of myelinated nerve fibers (MF) separated from the surrounding basement membrane (arrow) by astrocyte (glial) process (G). ($\times 20\ 000$)

MATERIALS AND METHODS

In the present study 8 adult albino rats (weighing between 200–250 g) were used. The animals were fed ad libitum and allowed free water supply. They were housed in cages, 4 rats per cage, under strict care and hygiene to keep them in healthy conditions. The animals were divided into two groups, 1 wpo group and 4 wpo group. They were deeply anaesthetized by diazepam and hypnorm mixture in a dose of 0.003 ml and 0.005 ml/10 g body weight respectively by intravenous injection obtained from Sigma Pharma Company, Cairo, Egypt. Optic nerve was exposed intracranially by a 3 cm long incision of the scalp along the mid saggital line. The right parietal bone was exposed by retracting the scalp. Three burr holes were made in the parietal bone along the parasaggital line about 1 mm from the mid saggital plane, using a dental drill (1 mm in diameter). Two other burr holes were made along a parasaggital line midway between the upper eyelids and the median saggital plane. The parietal bone between the holes was removed by using bone forceps. The intracranial portion of the left optic nerve was exposed by elevating the left frontal lobe (by gentle suction) and washing the cavity, using cold Hank's solution. The optic nerve was seen emerging from the optic foramen and passing posteriorly in the cranial cavity. A hole was made (by forceps pinching) in the meningeal membrane on the inferior surface of the frontal lobe. The hole was gently enlarged by micro scissor and the optic nerve was transected intracranially about 7 mm behind the eye ball. The cavity was filled by surgicell and the scalp was closed with 4/0 silk sutures and the animals were returned back to their cages.

Six animals (out of 8) survived this surgical procedure. Three of them were scarified one week post operatively (1 wpo group) and the other three were scarified 4 weeks post operatively (4 wpo group). The proximal stump of the transected optic nerves were isolated and trimmed into several pieces under a dissecting microscope. Fixation was done by 0.5% glutaraldehyde in 0.1 M phosphate buffer overnight, post fixed in OsO_4 for one hour. Gradual dehydration was done by using ascending grades of ethyl alcohol followed by embedding in araldite. Ultrathin sections (50–60 nm) were finally stained by lead and uranyl acetate salts (Stempak and Ward, 1964), processed for TEM.

(1) Central, (2) intermediate and (3) peripheral zones of the cut sections from the control and experimental animals were studied

RESULTS

One week after transection of the optic nerve (1 wpo group)

The central core of the transected nerve showed small bundles of sprout-like axons, particularly at the periphery (Figures 3 and 4). They were present close

to the blood vessels and some of them ran in different direction.

Longitudinally oriented axons were mainly containing smooth endoplasmic reticulum (sER) and microtubules. In some sections numerous dense core vesicles were also seen. Astrocytes, fibroblasts and collagen fibrils were seen in the vicinity of the astrocyte processes. A moderately wide intercellular space containing phagocytic cells with phagocytosed axonal, glial debris (filaments) and fragmented myelin were also seen. Some blood vessels with patent lumina, and intact endothelium were present in the central core of the nerve stump.

The intermediate zone (Figures 5 and 6) showed the presence of apparently intact axons and debris of a few degenerated cells. Many of the myelinated and the non myelinated degenerated axons appeared electron dense or swollen and most of them were organelle rich. Some were enclosed within an abnormally thin myelin sheath. There were many phagocytic cells that contained myelin debris and lipid droplets. Bundles of relatively small sized non myelinated axonal profiles were also seen adjacent to core zone. They were tightly packed together and contained microtubules and in some cases elements of sER and lysosome like dense bodies. As in the central zone sprouts were more frequently seen close to blood vessels than elsewhere. Astrocyte processes occasionally linked by adherent junctions or gap junctions at the intermediate zone. Astrocyte, cell bodies and processes were also seen here. Most of the blood vessels were having intact endothelial cells and patent lumina.

The peripheral zone (Figure 7) showed a picture similar to intermediate zone. Frequent non myelinated axonal sprouts could be seen in this zone. Normally appeared myelinated and degenerating fibers were observed. Swollen non myelinated axonal profiles, astrocyte processes were also seen in this zone.

Four weeks after transection of the optic nerve (4 wpo group):

The central zone (Figures 8 and 9) showed transverse section (TS) of bundles of non myelinated axonal sprouts containing microtubules and a few microfilaments. They were surrounded by thin cell processes resembling fibroblast processes. Collagen fibrils were present in the intercellular space. Few degenerated myelin sheaths and associated glial cell bodies were also seen.

The intermediate zone (Figure 10) showed a combination of presumptive axonal sprouts and large non myelinated axonal profiles. Several astrocytes were also seen in their vicinity. Phagocytic cells and fibroblasts were rarely seen in the sections of the 4 wpo group.

In the peripheral zone (Figures 11 and 12) bundles of axonal sprouts were seen. They were closely surrounded by astrocyte processes. These processes were joined together by adherent junctions. Few relatively normally appeared myelinated axons were also seen. Larger demyelinated axonal profiles were observed too.



Fig. 3. Electron photomicrograph of the central zone (core) of the proximal stump of the optic nerve, in the 1 wpo group, showing degenerative changes of axonal profiles (*), small regenerative axonal sprouts (arrow head), and large demyelinated axonal profiles. Astrocyte processes (A) extending between bundle of axons are also seen ($\times 44\,000$).



Fig. 4. Electron photomicrograph of the degenerative core in the 1 wpo group showing phagocytic cells containing myelin debris (M), fibroblasts (F), patent blood vessel (BV) in the intercellular space. Small bundles of putative sprouts (arrow) are seen close to astrocyte (A) ($\times 5\,000$).

DISCUSSION

Examination of the different zones and durations

Lack or even loss of the myelinated axonal profiles is an established sign of degeneration. Predominance of the regenerating non myelinated axonal profiles (sprouts) and the presence of phagocytic cells (macrophages) with the phagocytosed cell debris most probably myelin is also considered as signs of degeneration. The reappearance of apparently healthy myelinated axons is considered as a clear sign of healing which was evident peripherally than centrally in the 4 wpo group in the present study. A matrix of astrocytes with their intervening processes was also found in the 4 wpo group in the present study. Astrocytes might play a role in the regenerative and healing processes by providing a network in which sprouts are suspended. Other types of brain damage such as stab wounds, stroke and chronic injury by the accumulation of amyloid plaques also induce ectopic proliferation of various cell types such as microglia, astrocytes and endogenous precursors How-

ever, normally these precursors fail to generate neurons and instead contribute to the generation of glial scarring. Some of these endogenous precursors show the potential to generate neurons *in vitro* but somehow fail to do so upon injury *in vivo* (Kaslin 2008)

It is difficult to distinguish the axonal sprouts morphologically from pre-existing non myelinated axons. However, considering the fact that most of the small axons like profiles were clustered together without intervening cellular glial elements were non myelinated and between 0.1 and 1.4 μm in diameter compared with a diameter range of 0.2–2.0 μm for RGC axons in the optic nerve of normal adult rats (Fukuda *et al.* 1982). Beside that, the putative sprouts were very similar in size and ultrastructural characteristics to the axonal sprouts which grew into the zone of traumatic damage after transection of the intraorbital portion of the optic nerve. Most strong evidence in favour of these axonal profiles to be sprouts is the fact that they were completely absent from the degenerative core of the injured optic nerve in the 1 wpo group but were present in the

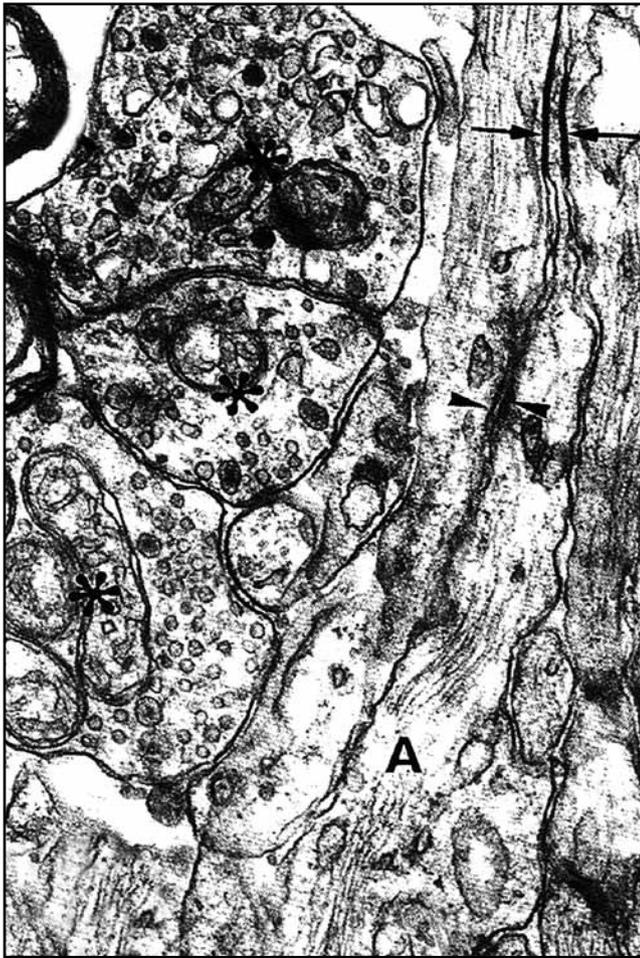


Fig. 5. Electron photomicrograph of the intermediate zone of the transected optic nerve 1 wpo group, showing several large axonal profiles (*). Axonal profiles are packed with synaptic vesicles, and mitochondria. These axonal profiles are lying adjacent to disrupted myelinated fibers (upper left side). Bundle of astrocyte processes (A) containing intermediate glial filaments and between them adherent junctions (arrow heads) and gap junctions (arrows) are evident ($\times 40\,000$).



Fig. 6. Electron photomicrograph of the intermediate zone in the 1 wpo group showing a bundle of regenerative axonal (demyelinated) sprouts (*) among abnormal irregular myelinated axons with disrupted myelin sheath (arrow). In vicinity an astrocyte process (A) is also seen that separates the bundle from the wall of a blood vessel (BV) ($\times 18\,000$).

4 wpo group in the present study. The presence of such axons as sprouts is considered to be logical. However, some of the larger axonal profiles, especially those containing numerous neurofilaments, and microtubules, in the intermediate zone and at longer survival times, in the peripheral zone as well, might be the surviving RGC axons from which myelin had been shed but which had not degenerated. Optic nerve transection induces apoptosis of RGC which is blocked by anti-apoptotic regimes and thus, in combination with blockers of axon-growth inhibitory signalling and promoters of axon growth may be a therapeutic formula for promoting sustained axon regeneration (Berry *et al.* 2008).

The degeneration within the proximal stump of the optic nerve after intracranial transection in the present study is most likely of ischemic origin. That is because, the necrosis was more marked centrally than peripherally i. e. away from the blood supply that comes from outside (intracranial). Also, the recovery started peripherally (nearer to blood supply). A minimal

degree of degeneration and necrosis occurs if appropriate measures are taken to protect the ophthalmic artery from damage during transection of the optic nerve. Misantone *et al.* (1984) performed intracranial crush lesions of the rat optic nerve using a jeweler's forceps. They were able to achieve complete transection of the optic nerve axons without disruption of the blood vessels. Their results revealed little indication of retrograde axonal degeneration up to 1-month post operatively within the proximal stump. Similar patterns of degeneration and necrosis have been reported by (Anderson 1970) who interrupted the branches of the circle of Willis which supply the intracranial part of the optic nerve and observed these changes in the nerve. This supports to the assumption that the necrosis in the central core of the optic nerve found in this study was of ischemic origin. Although the distal part of the proximal stump (toward the brain) became necrotic, the part of the optic nerve adjacent to the eye was spared presumably because it receives blood supply from the cen-

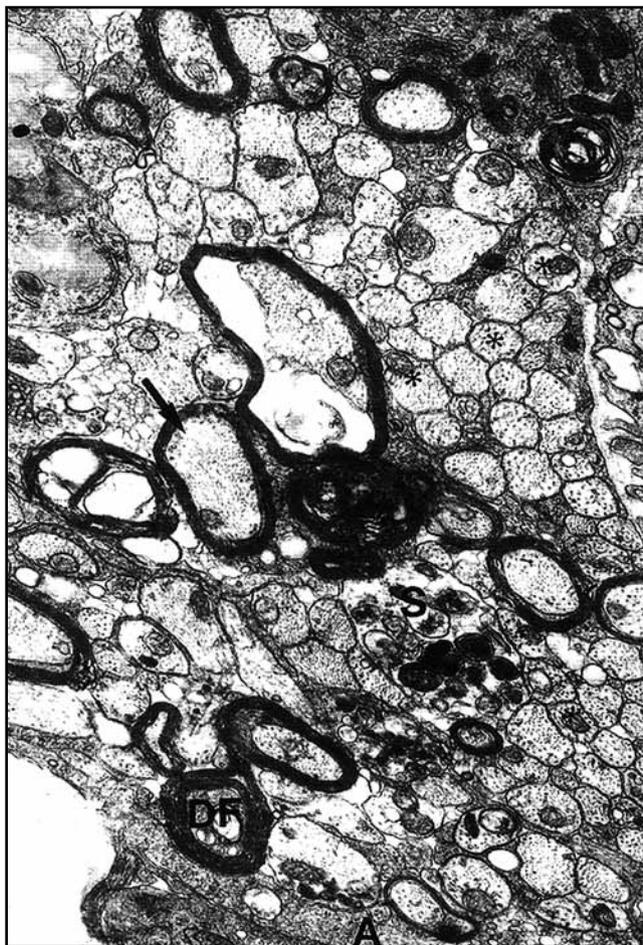


Fig. 7. Electron photomicrograph of the peripheral zone of the optic nerve in the 1 wpo group. Some of the myelinated axons are of relatively normal appearance (arrows); others are degenerating (DF), accompanied by numerous axonal sprouts (*). Swollen non myelinated axonal profiles (S) and astrocyte process (A) are also seen ($\times 10\,000$).

tral artery of the retina, which pierces the optic nerve just behind the eye ball (Grafstein & Ingoglia 1982; Richardson *et al.* 1982).

Comparison of the effect of intracranial and intra-orbital axotomy on RGC survival

No attempt was made to quantify the number of axonal sprouts formed following either intra orbital optic nerve transection (Hall & Berry 1989; Zeng *et al.* 1994) or intracranial optic nerve transection. Such a task would be exceptionally difficult because of the variable course of the axonal sprouts and the great variation in the number of sprouts in different parts of the injured nerves.

Axons cut close to the cell body show a greater regenerative response than those cut more distally, and axons may regenerate with greater vigor if they have been damaged some days previously (Verma *et al.* 2005). Axonal transport and immunohistochemistry done by Doster *et al.* (1991) have shown a positive correlation between the surviving retinal ganglionic cells and the extent of expression of growth associated

protein (GAP-43). These observations indicate that when the lesion is close to the eye, the response of the RGCs is stronger. For successful regeneration, the cut axon tip must first be remodeled to reform a new growth cone, a structure critical for subsequent axonal extension (Baas & Luo 2001). Even axons disconnected from their cell bodies *in vitro* can elaborate new growth cones at the site of lesion (Baas & Heidemann 1986), suggesting that most axons contain or are able to synthesize molecules needed for this process. Regenerating mammalian axons are tipped by a growth cone, and failure to make a new growth cone after axotomy would therefore make an axon unable to regenerate (Ziv & Spira 1998; Baas & Luo 2001). Most clinical injuries occur at some distance from the cell body, yet axonal regeneration may begin as rapidly as 1 day after injury (Pan *et al.* 2003). This, coupled to observations that growth cone regeneration in sensory and retinal axons *in vitro* can occur even when axons are surgically isolated from their cell bodies, suggests that the first step in regeneration does not have to wait the arrival of new proteins from the cell body (Baas & Heidemann 1986). Taylor *et al.* (2009) in their study has demonstrated that CNS axons synthesize proteins locally, maintaining a degree of autonomy from the cell body. However, axonal transport is increased after axotomy, and new proteins from the cell body will arrive in time to support continuing axon regeneration (Verma *et al.* 2005).

Astrocytes outside the cone of degeneration respond to intracranial optic nerve transection by undergoing hypertrophy, and by extending processes and migrating into the degenerative core. There is evidence from both *in vivo* and *in vitro* studies that astrocytes may play a role in the growth and regeneration of CNS neurons (Mathewson & Berry 1985; Gage *et al.* 1988). Astrocytes have been suggested to make and secrete neurotrophic factors that can subsequently support cell survival and axonal outgrowth (Banker 1980; Noble *et al.* 1984; Gage *et al.* 1988). In the mouse and rat visual system a variety of experimental paradigms promote survival of retinal ganglion cells (RGC) and optic nerve regeneration, probably through stimulation by neurotrophic factors (NTF) either directly, or indirectly through retinal astrocyte/Müller cell intermediary activation (Berry *et al.* 2008). In contrast to this astrocytes role has also been reported in the formation of thick cellular scar at the site of traumatic damage in CNS and thus contributing in the impairment of regeneration of the CNS (Reier *et al.* 1983; Anderson *et al.* 1989; Campbell *et al.* 1992).

Numerous astrocytes with their intervening processes have been found in the present study. Oligodendrocytes or their processes were not seen in the degenerative core in any of the group in the present study. The pattern of astrocyte response was very similar to that was found, following intraorbital optic nerve transection as reported by Zeng *et al.* 1994. This study has shown a strong association between the regenera-

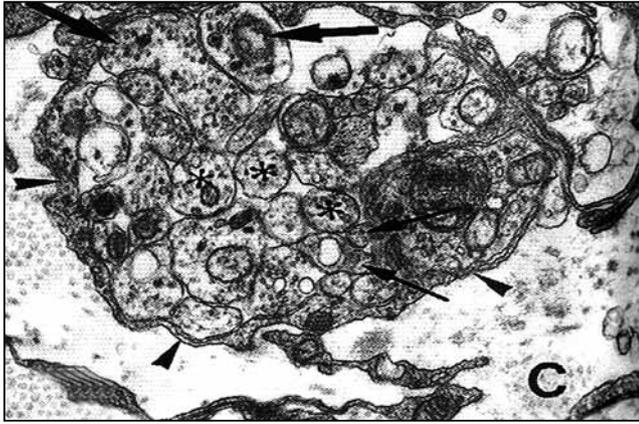


Fig. 8. Electron photomicrograph of the central zone of the transected optic nerve in the 4 wpo group, showing a bundle (arrow head) of non myelinated axonal sprouts profiles (*). The larger axonal profiles are containing many small clear vesicles and a few dense cored vesicles (thick arrows), while The small axonal profiles are containing microtubules and few microfilaments (thin arrow). Collagen (C) is present in the intercellular space ($\times 20\ 000$).

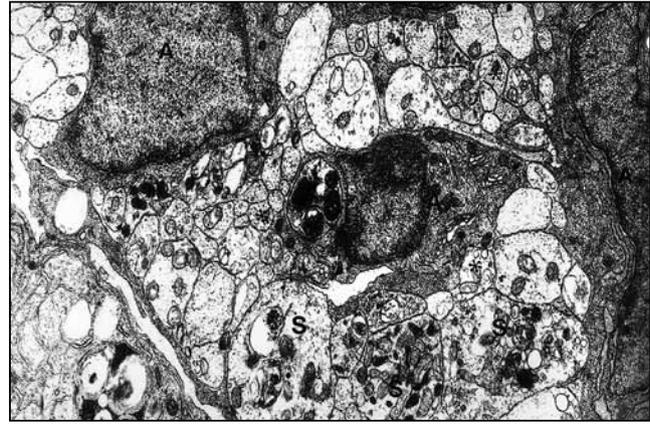


Fig. 10. Electron photomicrograph of the intermediate zone in the 4 wpo group showing many axonal sprouts (*) and a large non myelinated axonal profiles (S) containing many mitochondria and electron dense bodies. various astrocytes (A) are also seen ($\times 8\ 000$).

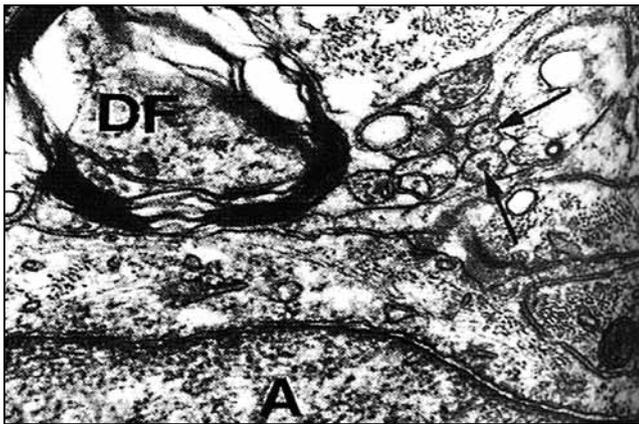


Fig. 9. Electron photomicrograph of the central zone in the 4 wpo group showing a few axonal spouts (arrows), and a degenerated myelinated fiber (DF) which is showing early signs of demyelination (disruption and separation of myelin sheath). Part of astrocyte nucleus is also seen ($\times 40\ 000$).

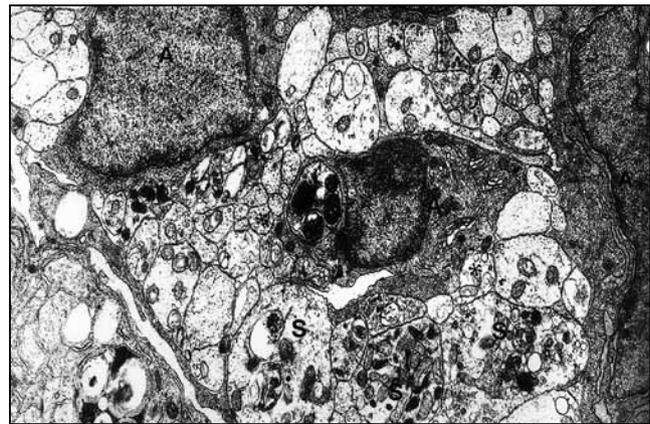


Fig. 11. Electron photomicrograph of the peripheral zone in the 4 wpo group showing different sizes of non myelinated axonal sprouts (*) and a few myelinated fibers of relatively normal appearance (arrow head). Astrocyte process (A) and larger degenerated profiles (arrows) are also seen ($\times 12\ 000$).

tive sprouts and the astrocyte processes. The present study supports the hypothesis that in the early phases of the response to the injury, astrocytes within the proximal stump of the optic nerve are not essential for the initial sprouting of axons. They may contribute as a helper for the elongation of some axons since they often become associated with growing sprouts (Weinberg & Raine 1980; Hall & Berry 1989; Campbell *et al.* 1992).

CONCLUSION

The interpretation of the present results of the different zones in 1 wpo group and 4 wpo group revealed marked signs of degeneration that were maximal in the central zone and decreased towards the periphery. In addition, degeneration was severe in the 1 wpo group and signs of recovery were obvious in 4 wpo group.

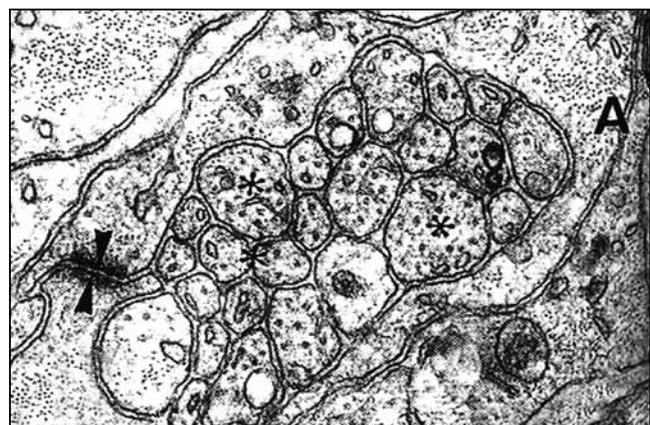


Fig. 12. Electron photomicrograph of the peripheral zone of the 4 wpo group showing a bundle of axonal sprouts (*) embedded in astrocyte processes (A). Adherent junctions (arrow heads) between the astrocyte processes are also seen ($\times 55\ 000$).

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