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Invited Review

Histopathological characterization of photochemical damage in nervous tissue

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Summary. This paper discusses histological and ultrastructural changes produced by dye-sensitized photoreactions in the central and peripheral nervous system. Particular attention has been given to morphological outcome in experimental models which reproduce widespread clinical pathologies, e.g. stroke, spinal cord injury and peripheral neuropathy. Evaluation of structural alterations may not only help to characterize the evolution of these disease processes but also allow us to study possibilities of therapeutic intervention.

Key words: Dye-sensitized lesions, Brain, Spinal cord, Peripheral nerve, Histology

Introduction

Alteration of cellular structure and function after generation of reactive oxygen species following illumination of photosensitizing dyes is a principle which has been used for many purposes in neurobiological research (for review: see Pooler and Valenzeno, 1981; Spikes, 1991). The majority of available studies are based upon external application of the dye or by its injection into neuronal cells. Such treatment may - depending on the conditions - either result in nerve stimulation or inactivation (Pooler and Valenzeno, 1981). Dye-sensitized reactions thus offer an elegant possibility to interfere with physiological mechanisms in nervous tissue and to study secondary structural phenomena such as neurodegeneration and reactive neuroplasticity.

Photochemical models which closely resemble human disease are limited. The introduction of a non-invasive brain infarction model based on Rose Bengal-sensitized photothrombosis (Watson et al., 1985) stimulated the study of stroke pathology and therapy. A slightly modified procedure has been used to study structural and functional cerebral protection with the calcium entry blocker flunarizine (Van Reempts et al., 1987; De Ryck et al., 1989). Other investigators used photothrombosis to study drug effects on ischemic neurodegeneration in the retina (Mosinger et al., 1991) or to study drug effects on spinal cord necrosis and behavioural abnormalities (Prado et al., 1987; Pencalet, 1993).

In contrast to procedures in which neuronal degeneration indirectly results from photochemically initiated ischemia, it is also possible to induce direct degeneration of nerve cells. Recently, we introduced a new model of reversible sciatic nerve demyelination with minimal axonal degeneration based on photosensitization of topically-applied Rose Bengal (Van Reempts et al., 1993).

The present paper will focus on histopathological data obtained in experimental models of stroke, spinal cord injury and peripheral neuropathy. Special attention will be given to temporal progresssion of the lesion.

Photochemical modification of cell membranes

The combined action of visible light and photosensitizing molecules upon biological membranes and the mechanisms which underlie their modification or destruction have been thoroughly investigated (for review: see Foote, 1976; Pooler and Valenzeno, 1981; Valenzeno, 1987). To be effective, a sensitizer molecule has to be associated with a hydrophobic cell membrane environment. Of the halogenated fluorescein compounds, Rose Bengal is the most efficient photomodifier. In particular for nerve cell inactivation, it is about 35000 times more effective than fluorescein (Valenzeno, 1987). This makes Rose Bengal very attractive for *in vivo* studies, since it can be used at much lower plasma concentrations (Watson et al., 1985). Once bound to a cell membrane, either the surface membrane or intracellular membrane components, photons are absorbed by the sensitizer during illumination at an

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appropriate wavelength. Active quenching of the excited triplet sensitizer by molecular oxygen creates highly excited singlet oxygen which induces oxidation of susceptible membrane components (Foote, 1976; Pooler and Valenzeno, 1981).

Although the surface membrane is an important target and since cellular function largely depends upon its permeability and transport properties, it is not excluded that intracellular structures are modified as well after penetration of the sensitizer in the cytosol. This means that virtually any cell function can become compromized, depending on where and in which concentration the sensitizer has been applied and how much light reaches the cell. A chain of secondary reactions can be expected in complex and vulnerable cellular environments such as the nervous system.

In the following paragraphs a number of such experimental approaches will be discussed.

Photothrombotic cerebral infarction

An important part of human stroke cases result from thrombotic occlusion of major blood vessels. Watson et al. (1985) developed a minimally invasive model of inducing cerebral infarction after photochemically-initiated thrombosis in rat cortex. The principle had been used earlier by Rosenblum and El-Sabban (1977) who studied platelet aggregation in superficial cerebral microvessels after photoexcitation of sodium fluorescein. Several modifications have been introduced since then, including the use of cold light either unfiltered (Van Reempts et al., 1987) or filtered at 560 nm (Grome et al., 1988), use of a laser beam to selectively occlude the middle cerebral artery (Watson et al., 1987; Nakayama et al., 1988), intracranial implantation of polymethylmetacrylate frames containing an optic fibre to allow infarct production in conscious rabbits (Van Rossem et al., 1992a), stereotaxic implantation of small diameter cold light fibres to

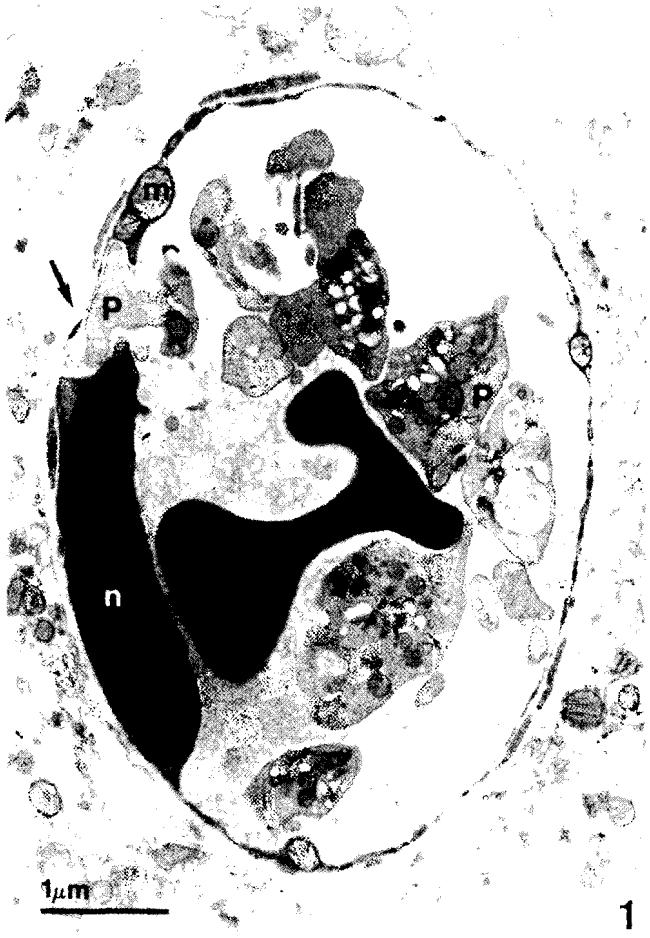


Fig. 1. Electron micrograph of a small cortical vessel showing photochemically-induced endothelial damage. Platelets (P) migrate to sites where endothelial cells become detached from the basal lamina (arrow) whereafter a thrombus progressively develops. m: mitochondria; n: nucleus; E: erythrocyte.

Fig. 2. Detail of a 100 µm section through rat cortex 30 min after induction of photothrombosis. After i.v. injection of 1nm gold suspension the thrombotic area (T) can be recognized by the absence of tracer in the microvascular bed. The surrounding parenchyma is adequately perfused but in the proximal perithrombotic rim several vascular leaks are prominent (arrows).

produce microinfarctions (Van Reempts et al., 1989b), lesioning of accurately defined anatomical sites, e.g. sensorimotor cortex to predict functional outcome (De Ryck et al., 1989) or inducing vascular thrombosis in the rat retina as a simple alternative to the CNS (Mosinger and Olney, 1989). Evolution of injury in these experimental models has been extensively investigated. We will illustrate the morphological aspects as they appear in hypertensive rats subjected to photochemical stroke (Van Reempts et al., 1987).

Intravenous injection of Rose Bengal and subsequent illumination of the brain tissue either through the intact skull or via a microfibre stereotaxically inserted in the parenchyma results in a well delineated progressively expanding infarction that can be easily recognized in the light microscope after staining with standard histological procedures (Watson et al., 1985; Van Reempts et al., 1987, 1989b; Grome et al., 1988). During the first minutes following sensitization endothelial membranes became disrupted and numerous platelet aggregates were formed within cortical vessels (Watson et al., 1985; Dietrich et al., 1987b) (Fig. 1). This resulted in thrombus formation, local ischemic reaction and irreversible infarction within 24 h. The size of the thrombus, which was dependent on the diameter of the illuminated area and the depth of light penetration, was significantly smaller than the final infarction. This is indicative of a progressive expansion of the lesioned area (Van Reempts et al., 1987; Grome et al., 1988). This phenomenon most probably resides at the peripheral margin of the initial thrombus where edema and vascular leakage are prominent within less than 15 min (Dietrich et al., 1987a; Van Reempts et al., 1987) (Figs. 2, 3a,b). Within 4 h the irradiated tissue became characterized by congested vessels, severely coagulated neurons, dilated glial cells and spongious parenchyma (Fig. 3c). At the edges of the congested zone neurons appeared more damaged when compared to the central part. In the latter, oxygen tension rapidly dropped to zero levels (Van Rossem et al., 1992b). Such condition of no flow is regarded as less harmful (Siesjö, 1981). However, in a peripheral rim around the thrombotic core one could find open vessels and viable neurons. Glial cells, which are supposed to maintain ion homeostasis, in this area appeared extremely swollen (Van Reempts et al., 1987) (Fig. 3d).

Sites of extravasation coincided with areas of reduced basophilic staining reaction. Such weakly stained areas might be related to regions of reduced light penetration, not sufficiently intense to cause platelet aggregation and consequent thrombosis, but strong enough to induce minor endothelial injury and hence development of vasogenic edema. In such a way also the photosensitizing dye might invade the brain tissue and cause direct toxic effects to glial cells and neurons. However, such a mechanism is questionable for several reasons. Firstly, extravasation and the prevalence of edema close to a thrombotic core, are initially more pronounced in superficial cortical regions (Van Reempts and Borgers, 1990; Lanens et al., 1993). Nevertheless, expansion of the infarct does not only occur laterally but also in the direction of deep cortical layers. Secondly, reactive oxygen species have a very short lifetime (Valenzeno, 1987) and hence their aggression is limited to the duration of light exposure or the period immediately thereafter. However, it has been shown that extravasation and glial edema remain visible around an expanded infarct for more than 24 h (Van Reempts et al., 1991; Van Bruggen et al., 1992; Lanens et al., 1993). Finally, these leaky margins appear very well delineated (Fig. 3a,b), whereas light intensity is supposed to decrease gradually. Therefore it can be concluded that phenomena secondary to thrombosis rather than direct dye-induced cytotoxicity may be held responsible for histological changes observed in the progressively expanding perithrombotic region. Further evolution of the focal ischemic lesion is mainly characterized by infiltration of macrophages and proliferation of glial cells around the ischemic core (Watson et al., 1985; Grome et al., 1988; Meyers et al., 1991; Verlooy et al., 1993).

Blood-borne substances released from the site of vascular thrombosis have been shown to acutely affect vascular permeability (Dietrich et al., 1988). It is conceivable that regions with increased tracer extravasation are the equivalent of increased water content which has been measured in the irradiated area (Dietrich et al., 1987a; Grome et al., 1988) and that they correlate with diffusion-weighted NMR images obtained from a similar cortical lesion, i.e. cortical areas which remain well perfused but leaky and which distinguish the edematous non-necrotic periphery from the infarcted core (Van Bruggen et al., 1992). On the other hand, T2-weighted images at 24 h reveal an expanded area of hyperintensity which is reversible (Lanens et al., 1993). It is not excluded that the latter area coincides with both the region of extravasation and a region of glial reactivity and cytotoxic swelling which extends far beyond the leaky rim (Meyers et al., 1991; Van Reempts et al., 1991). Ongoing experiments comparing NMR images with the histological picture in the same animal are in favour of this statement.

In contrast to observations after middle cerebral artery occlusion which indicate that alterations in the peri-infarct zone are comparable to hypoglycemic brain damage (Nedergaard, 1987, 1988), the main alterations in the photothrombotic stroke model at the infarct periphery consist of extreme glial swelling. This can be explained by a slow initiation of ischemia during development of the thrombus which creates a temporal condition of low perfusion and hence facilitates formation of lactic acidosis. The latter promotes severe edema of the glial compartment (Plum, 1983). Whereas the core will become mainly infarcted as a result of slowly initiated but irreversible congestion, the margins will suffer secondarily from mechanical compression by perivascular endfeet (Dietrich et al., 1987a).

It remains a matter of debate whether such a picture of edematous glial cells encompassing

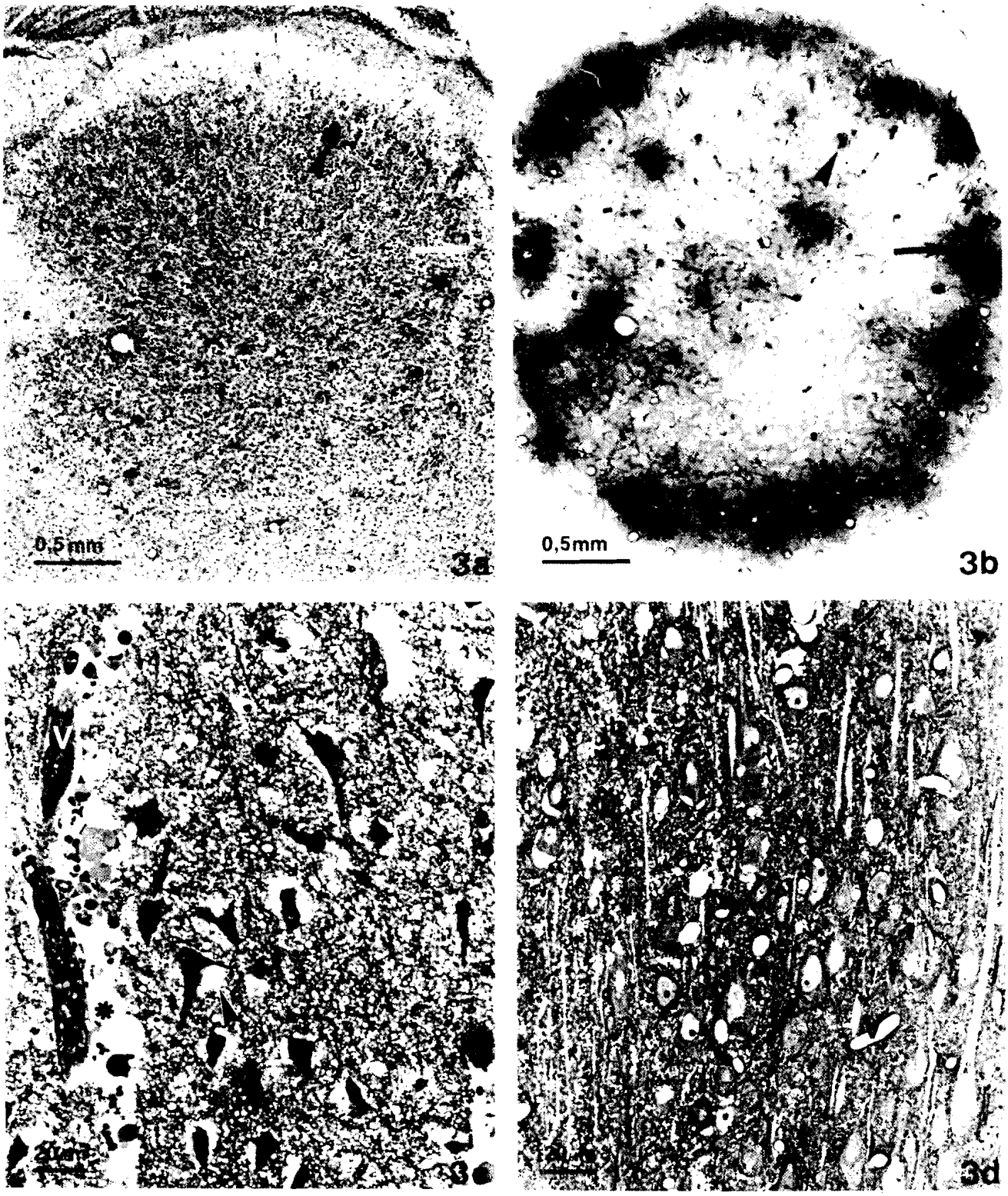


Fig. 3. Photochemical lesion in rat brain 1h (a,b) and 4h (c,d) following start of illumination. Consecutive horizontal 100 μm sections stained with azure-eosin (a) or silver-enhanced to demonstrate 1 nm gold tracer (b) show a clearly delineated edematous rim (a, white arrow) correlating with the area of tracer extravasation (b, black arrow). Blood vessels in the thrombotic core appear congested (arrowheads). Toluidine blue-stained 2 μm section in the core (c) is characterized by thrombotic occlusion of vessels (V), neuronal necrosis (arrow), perivascular (asterisk) and perineuronal (arrowhead) glial swelling. In the peri-infarct border (d) vessels are open (V), neurons are morphologically well-preserved (arrow) and glial cells appear extremely swollen (arrowhead).

morphologically intact neurons, can be regarded as the morphological substrate of the ischemic penumbra. This so called area at risk has been defined as a condition of the ischemic brain tissue with flow values between an upper threshold of electrical failure and a lower threshold of ion pump failure (Astrup et al., 1981). The fact that such an area is a dynamic physiological phenomenon makes it difficult to demonstrate it morphologically. Bilateral comparison of CBF in concentric areas around a photochemical infarction by means of non-radioactive microspheres, which can be easily localized in the light microscope, showed a gradual increase of flow from the edge of the infarct core towards distant areas. In between 2 and 24 h flow still remained less than 50% of the contralateral hemisphere. No quantifiable flow region under penumbra thresholds was found at any time point between 1 and 24 h, that could predict subsequent irreversible infarction (unpublished results). Possibly the flow-related penumbra is a very small rim, continuously moving outward from sites where vasogenic edema and cytotoxic glial swelling become sufficiently expressed to induce secondary ischemia by mechanical compression of microvessels.

Several attempts have been made to further characterize the area at risk adjacent and remote from the site of the primary thrombotic focus. Such regions showed different degrees of glucose hypermetabolism at 4 h after photothrombosis and diffuse hypometabolism at more distant sites (Dietrich et al., 1986). More detailed metabolic mapping at cellular level in this model has been obtained by localizing immediate early gene (IEG) immunoreactivity (Gass et al., 1992). In this study it has been shown that brain regions with IEG induction do not necessarily coincide with areas of hyper-metabolism. Such proteins are rapidly induced in the entire ipsilateral cortex with exception of the thrombotic focus. Increased expression of FOS B between 3 and 8 h confirms that the peri-infarct area still contains viable neurons capable of gene induction and protein synthesis (Gass et al., 1992). The study supports the concept of a cortical spreading depression-like mechanism of IEG induction by focal ischemia. Spreading depression has been observed in the peri-infarct zone after middle cerebral artery occlusion (Nedergaard, 1988). In photothrombotic stroke the profile of spontaneous and induced spreading depressions where shown to be a valid diagnostic tool

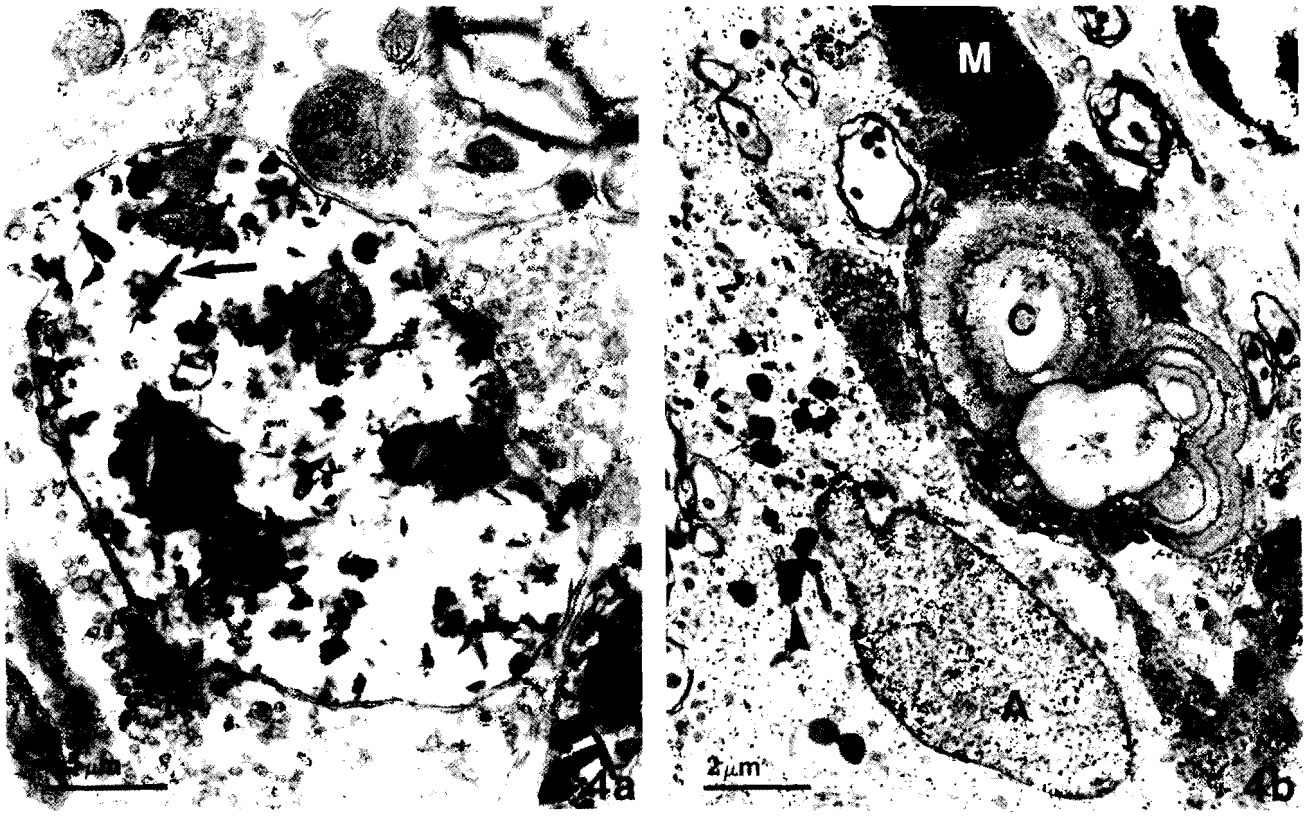


Fig. 4. Ultrastructural appearance of retrograde damage in rat thalamus after photothrombotic infarction of the sensorimotor cortex. One week after the insult **(a)** calcium deposits appear in several neuronal processes in the form of apatite crystals (arrow). After 3 weeks **(b)** they evolve towards large calcareous concretions. Immature forms are found inside glial cells (arrowhead), whereas giant lamellar calcifications (C) are located both intra- and extracellularly. Histochemical staining of purine nucleoside phosphorylase, shows increased amounts of reaction product (tiny black precipitates) in microglial cells (M) and astrocytes (A).

for the penumbra (De Ryck et al., 1992).

Depending on the anatomical localization of the infarct, retrograde or anterograde damage can be expected in distant structures. This we could demonstrate in the photothrombotic stroke model after lesioning of the sensorimotor cortex (De Ryck et al., 1989). Retrograde damage in thalamic nuclei is characterized by progressive calcium accumulation and severe gliosis (Van Reempts et al., 1989a). One week after the induction of photothrombosis calcium appears in high amounts in neuronal processes in the form of apatite crystals (Fig. 4a). The picture evolved after 3 weeks towards formation of large calcareous concretions, which could be easily recognized in the electron microscope (Fig. 4b) and which showed positive reaction when stained with alizarin red. Increased glial reactivity was found in the same structures from one week onwards. This could be nicely demonstrated with a histochemical method for localization of purine nucleoside phosphorylase activity (Van Reempts et al., 1989a) (Figs. 4b, 5). Such findings are not restricted to photochemically-induced infarction but also occur after severe insults such as cortical ablation (Cooper et al., 1984), and hypoxia-ischemia or kainate injection (Van Deuren et al., 1992). Their localization may offer additional information about secondary phenomena resulting from stroke.

Fig. 5. Ipsilateral damage in rat ventroposterolateral thalamic nucleus differentiated by enzyme staining for purine nucleoside phosphorylase, 3 weeks after photothrombotic infarction of sensorimotor cortex.

Spinal cord injury

Platelet aggregation and edema formation appear to be early phenomena after traumatic spinal cord injury (Goodman et al., 1979) and as such photothrombotic spinal cord infarction may be useful to investigate this secondary component of the injury process (Prado et al., 1987). A minimally invasive method which does not require laminectomy and produces graded spinal cord infarction in the rat has been described (Watson et al., 1986; Prado et al., 1987). By varying illumination times, the authors created reproducible damage to selective white matter tracts resulting in behavioural and electrophysiological deficit. Photoinduction times of 30 sec already revealed necrosis of the dorsal columns with acute conduction block which recovered by 8 weeks. Irradiation times of 10 min resulted in complete necrosis of almost the entire cord thickness with no improvement of behavioural deficit and conduction block within the 8-week observation period. Use of Erythrosin B as photosensitizer instead of Rose Bengal, which has a lower excitation efficiency at a wavelength of 514 nm, led to production of cystic lesions which in humans are known to further develop into post-traumatic syringomyelia (Cameron et al., 1990). Histopathological examination at 1 week showed a necrotic core surrounded by a vacuolated neuropil evolving at 1 month towards a large central cavity. Severe motor impairments recovered by 1 week but remained different from control. Hyperactive responses to sensory stimulation remained present up to 28 days. In our experiments with Rose Bengal-induced thrombotic spinal cord injury the structural appearance of the cord showed a highly variable degree of damage (unpublished results). This was true after testing different conditions of dye concentration (20-40 mg/kg), cold light irradiation (10-30 min) and survival (1 h-1 month). We concluded that with standard fibres it was extremely difficult to develop reproducible lesions which were sufficiently small to allow morphological analysis. Even after minor aggressive challenge, large cavities were formed which made detailed histological evaluation and infarct size quantification almost impossible (Fig. 6a). As an alternative we used a small-sized optic fibre, unilaterally placed on top of the dura between adjacent vertebrae. The resulting small infarction (Fig. 6b-d) could be easily delineated and studied light- and electron-microscopically but was not sufficient to induce marked neurological deficit.

Peripheral demyelination

The above-mentioned experimental models are based upon indirect ischemic challenge of the nervous tissue by photothrombosis. Neuronal damage can also be directly produced *in vivo* by topical application of the sensitizing dye combined with focal illumination. We have used this principle to induce acute demyelination of the peripheral nervous system with relative axonal sparing (Van Reempts et al., 1993). The progression of light microscopic and ultrastructural changes was followed over 1 month. Early inflammatory response and disintegration of myelin sheaths were present at 4 h. Macrophages became directed to disrupted myelin fragments which resulted in complete denudation of axons in and distal to the irradiated area after 1 week (Fig. 7a,b). Spontaneous remyelination was observed within 1 month (Fig. 7c,d). All animals showed rapid functional deterioration which spontaneously recovered. There was no clear correlation between functional outcome and progression of de- or remyelination.

Ongoing studies have shown that in a similar way it is possible to injure the dorsal fascicles of the spinal cord. A clear distinction could be made between the morphological appearance of demyelinated dorsal fascicles (ensheathed by oligodendroglia) and spinal nerves originating at the irradiated area (ensheathed by individual Schwann cells) (Fig. 8a,b). This makes the model attractive for the study of certain pathogenetic aspects of peripheral as well as central demyelinating diseases, e.g. Guillin-Barré syndrome or multiple sclerosis.

Importance for pharmacology

The use of *in vivo* photochemical models to produce

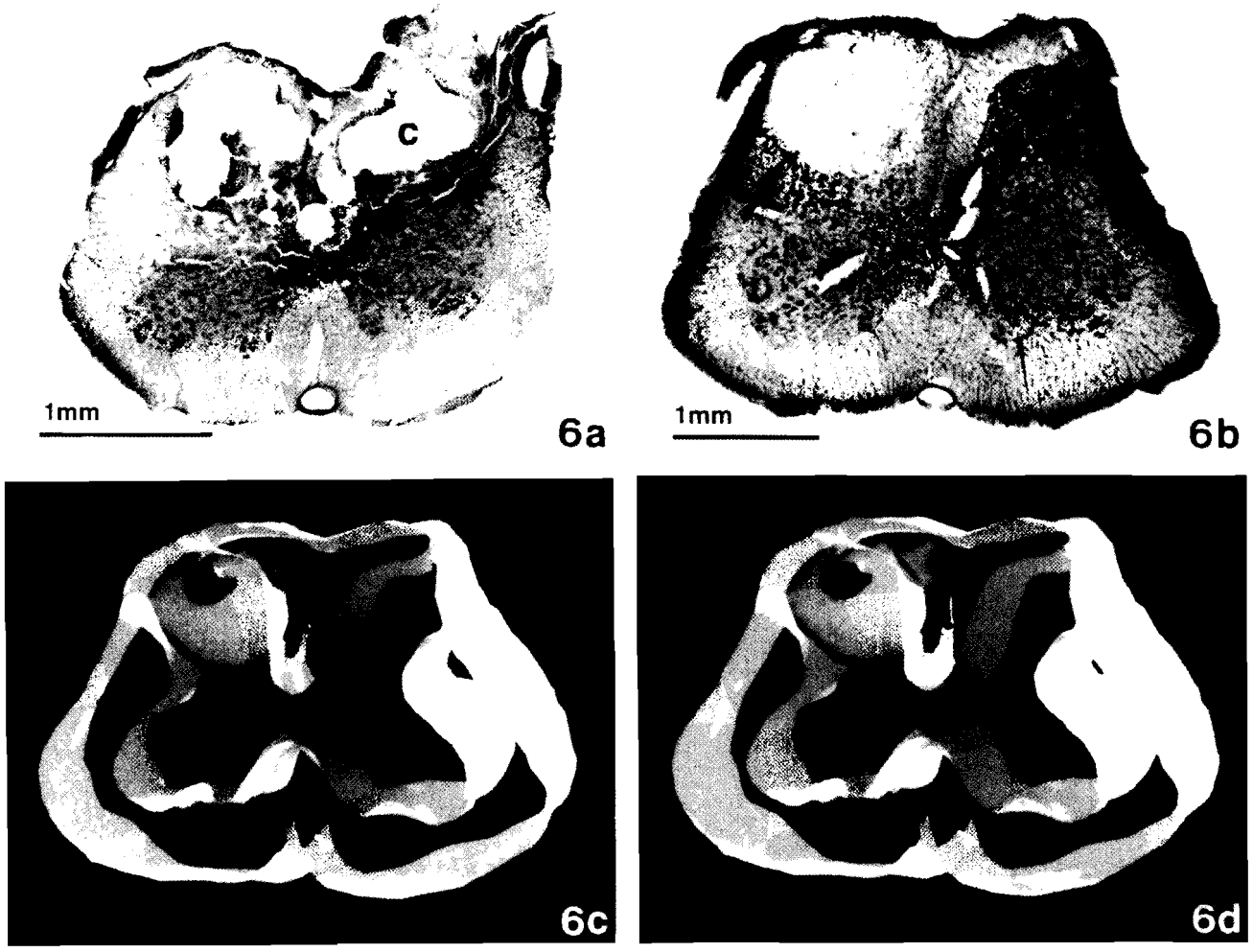
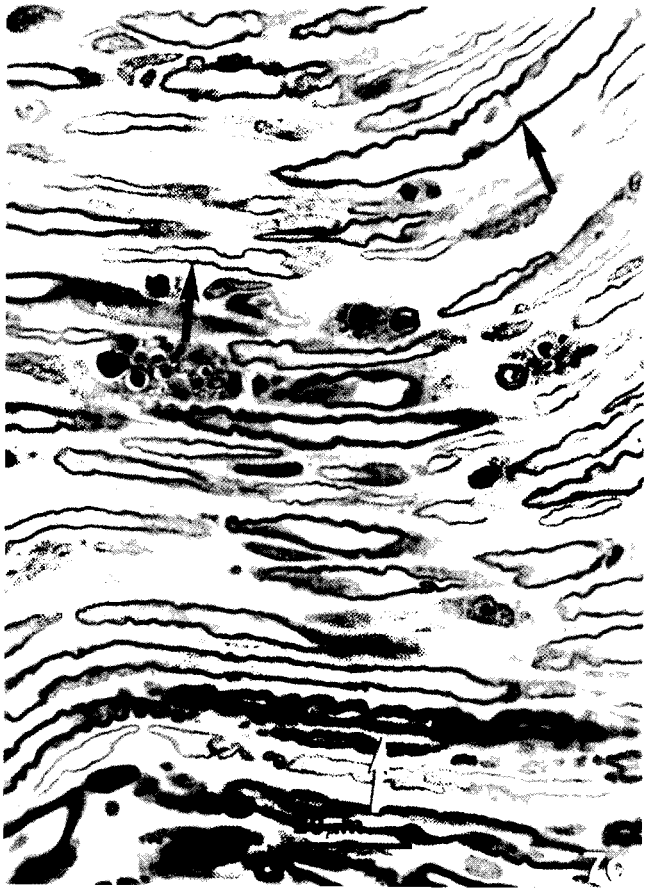
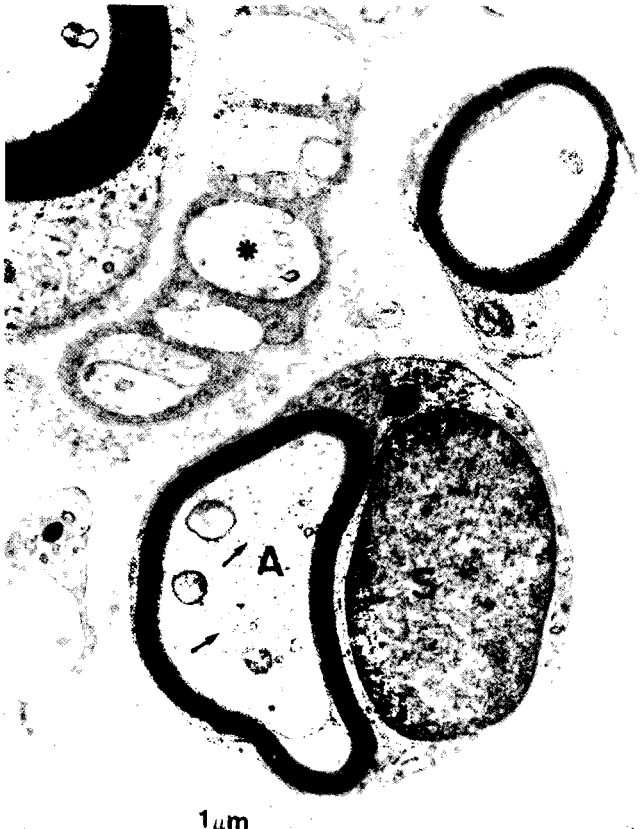


Fig. 6. Rat spinal cord injury at level L2, 24h after photothrombotic challenge. Illumination with a large-sized optic fibre (3 mm diameter; a) produces deep bilateral infarction with large cavities (c) in white and grey matter. Small-sized fibres (0.2 mm diameter; b) unilaterally placed upon the dura, result in small reproducible infarctions, mainly located in the grey dorsal horn (arrow). Three-dimensional reconstruction of such lesions from serial histological sections (c,d) facilitates topographical localization on stereo pairs of pictures.





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damage in the central and peripheral nervous system not only offers the possibility to examine pathogenetic processes which play a key role in certain disease processes but also permits evaluation of pharmacological therapy.

Infarct size, measured in hypertensive rats at 4 h following thrombotic stroke was significantly reduced after oral pretreatment with the calcium entry blocker flunarizine (Van Reempts et al., 1987). In the same model it has been shown that posttreatment with flunarizine prevented proprioceptive and tactile placing deficits after lesioning the hindlimb sensorimotor neocortex (De Ryck et al., 1989).

Ischemic damage in the retina could be reduced by NMDA antagonists (Mosinger et al., 1991). Postischemic induction of c-FOS and c-JUN in photochemically challenged rat cortex, was largely reduced in animals pretreated with MK-801 (Gass et al., 1992). Posttreatment of rats with the NMDA antagonist thienylphencyclidine after photochemical spinal cord

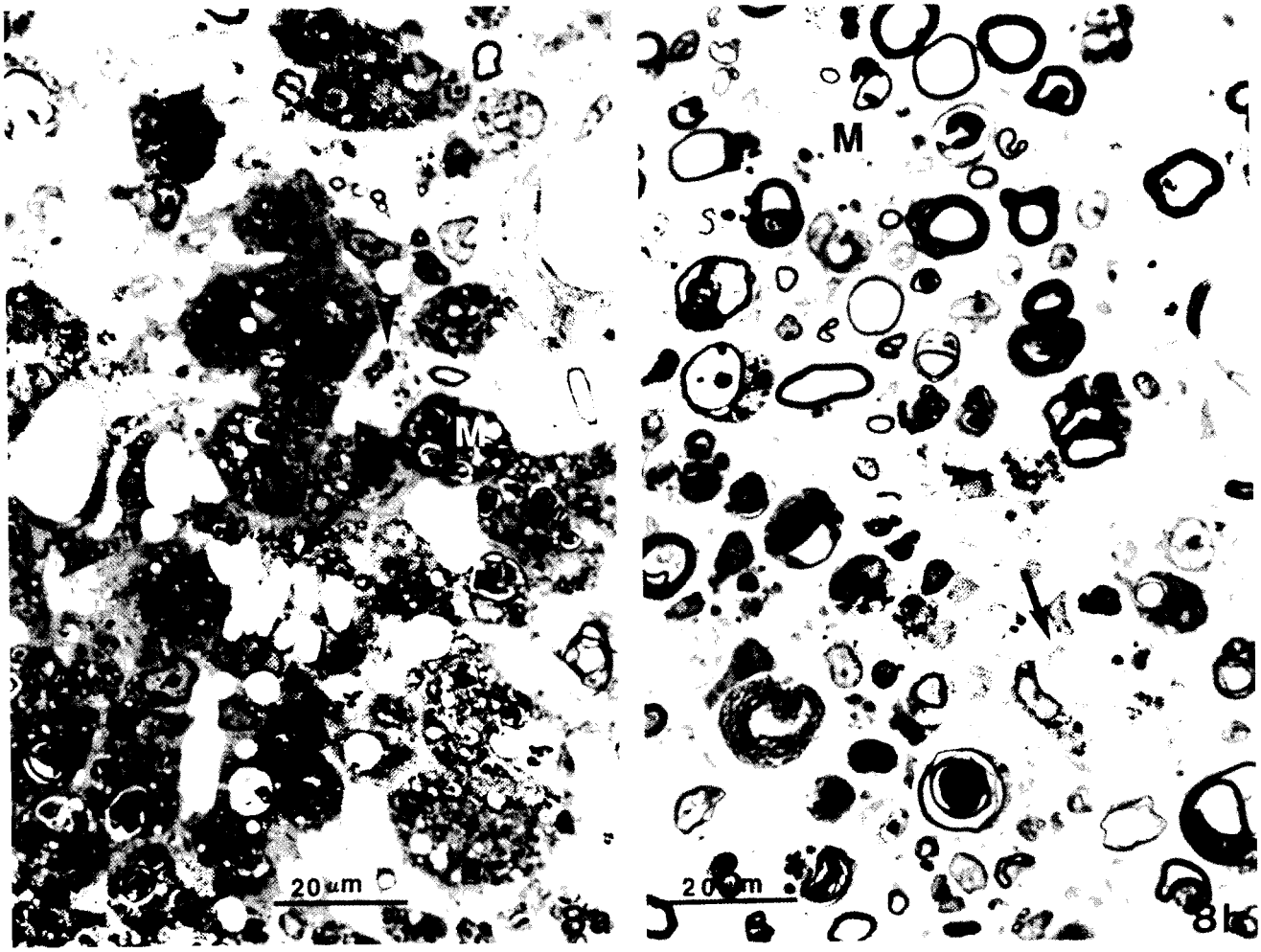

Fig. 8. Photochemical demyelination of rat spinal cord 5 days after intrathecal injection of Rose Bengal and subsequent illumination between two adjacent vertebrae at L2. Transverse 2 µm sections of dorsal fascicles **(a)** show groups of well preserved but denuded axons (arrow) surrounded by a lot of phagocytosing macrophages (M) and proliferating oligodendroglia (arrowhead). Spinal nerves branching at the irradiated site **(b)** show individual demyelinated axons (arrow), macrophages (M) and proliferating Schwann cells (arrowhead).

Fig. 7. Light microscopic (a,c) and ultrastructural (b,d) appearance of photochemical demyelination with axonal sparing of rat sciatic nerve after topical application of Rose Bengal. In the illuminated area at 5 days (a) the majority of axons lost their myelin sheath (arrows). They are surrounded by macrophages (arrowhead) and proliferating Schwann cells (double arrow). Deeper axons appear normal (white arrow). The ultrastructural detail (b) shows a macrophage (M) which invades redundant basal lamina (arrowheads) and contains several phagocytosed myelin fragments (asterisk). The denuded axon (A) is devoid of microtubules but contains abundant neurofilaments. One month after challenge (c) axons in the illuminated area show spontaneous remyelination (black arrows). Newly-formed sheaths are considerably thinner than those which remained unaltered (white arrow). The ultrastructural picture (d) shows remyelinated axons (A) surrounded by a Schwann cell (S). The axoplasm again contains normal amounts of microtubules (small arrows). Unmyelinated axons (asterisk) remain well preserved).

injury resulted in behavioural, electrophysiological and structural protection (Pencalet et al., 1993). These studies stress the important role of calcium and NMDA-dependent mechanisms in the pathogenesis of thrombotic stroke. In addition, they show the usefulness of these minimally invasive photochemical models to search for adequate pharmacoprotection.

Conclusions

Photosensitized membrane peroxidation has been shown to be a valuable tool for the experimental study of histopathological changes, functional outcome and pharmacological protection in central and peripheral nervous system disease. Apart from *in vitro* challenge of single neurons, it is possible to induce in a minimally invasive way graded damage to cerebral, spinal or peripheral neurons, either directly by topical application of the photosensitizer or indirectly by inducing thrombotic ischemia. The most interesting feature in such approaches is the possibility for exact anatomical positioning of the lesion and in such a way to predict functional outcome or localize distant areas where secondary damage can be expected. The use of light sources which are placed at a certain distance or just on top of the tissue avoid artificial damage but even after intracerebral insertion of small fiberoptics, photochemically-induced changes can be easily differentiated from mechanical damage. The initial changes are located at the level of the cell membrane to which the photosensitizing dye will bind first. The final outcome is therefore largely dependent on the specific function and vulnerability of the sensitized cell type. As far as cells or structures can be impregnated by the sensitizer and are accessible for the light beem, a lot of interesting paradigms can be proposed. Histological assessment can thereby add valuable information to understand pathophysiological mechanisms leading to human disease.

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