Growth and Myelination of Goldfish Optic Nerve Fibers after Retina Regeneration and Nerve Crush

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Z. Naturforsch. **33 c**, 988–996 (1978); received August 17/September 8, 1978

Retina Regeneration, Axon Regeneration, Goldfish Visual System, Synaptogenesis, Myelogenesis, Oligodendroglia

Axonal regeneration in the optic nerve and tectum of the goldfish was studied both after retina regeneration and nerve crush. The retina regeneration was evoked by ouabain-induced damage of at least the ganglion cells and cells of the inner nuclear layer. The necrotic retinal neurons are substituted by mitotic processes in the outer nuclear layer and the marginal growth zone at the ora serrata. The axons of these newly developed retina ganglion cells grow through the degenerating, but mechanically undamaged, optic nerve into the tectum, establishing there synaptic contacts already 16 days after the intraocular ouabain-injection. The fibers were myelinated at first in the tectum, later on in the optic nerve. Thus, the myelination process proceeds in retrograde direction. About 60–80 days after injection the myelination has become nearly normalized. On the contralateral side of the same animal, the optic nerve was crushed near the eye-bulb. The axons of the original retina ganglion cells grow out into the degenerating optic nerve and tectum. They also find synaptic contacts and are myelinated in retrograde direction, but to a much lesser extent than the axons of the regenerated retina ganglion cells. An axonal factor is discussed, which would influence the oligodendroglial myelination activity. The effectiveness of this factor is probably dependent on the neuronal age and suggested to be triggered by the establishment of synaptic contacts.

Introduction

Regeneration of the optic nerve fibers after crush is well known in fish and amphibia [1–6]. After crush of the nerve there is a time delay of 4–6 days, after which the nerve fibers sprout and grow out into the degenerating distal stump [7]. RNA- and protein synthesis in the perikarya of the retina ganglion cells [2, 8, 9] and the outgrowing of their axons after explanting the retina *in vitro* [10] are stimulated. After arrival in the tectum, the fibers make synaptic contact in the stratum opticum or in the stratum fibrosum et griseum superficiale with postsynaptic cells, establishing the restitution of visual function. It follows a remyelination of the optic fibers by the oligodendroglia [1, 5]. The remyelination pattern is known to be incomplete for several months, obviously this remyelination process is much slower than the process of ontogenetic myelination.

I have investigated both the fiber regeneration mode after nerve crush and after ouabain-induced degeneration and following regeneration of the retina. Retina regeneration in fish occurs as a very well reproducible phenomenon in contradiction to the statement of Walls (1942; cited after Locket [11]) and, more recently, of Schmidt *et al.* [12]. But surprisingly, it is nearly uninvestigated in fish, in contrast to the retina regeneration in amphibia (for review see Reyer [13]). Lombardo [14] has described the retina regeneration in a cyprinid teleost after partial surgical ablation of different retina regions. After degeneration of the goldfish retina by intraocular ouabain-injection [15] the optic nerve undergoes Wallerian degeneration [16], and the retina regenerates in the following time, both by increased proliferation of the marginal growth zone and, if remained intact, by mitogenic activity in the outer unclear layer [17–19]. About 6–8 days after ouabain injection new ganglion cell bodies have established, sending their axons into the degenerating optic nerve.

Thus, it is possible to compare the regrowth of crushed axons from the undamaged, original retina on the one side with the outgrowth of newly developed retina ganglion cell axons through the mechanically undisturbed nerve of the contralateral side of the same animal.

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Materials and Methods

Ouabain (Serva; 10 µl 10^{-5} M) was injected into one eyebulb of the goldfish *Carassius auratus* (about 12 cm body length). The contralateral optic nerve was crushed directly behind the eyebulb by means

of fine watchmaker forceps for about 2 sec. During the operation the animals were anaesthetized by 0.02% MS 222 (Sandoz). The experiment was finished after 8, 14, 16, 19, 28, 29, 38, 47, 57, 73 or 85 days. The animals were decapitated, the retina and the optic nerve and tectum were dissected out and fixed in 2% glutaraldehyde, buffered with 0.1 M cacodylate. After washing in buffer containing 0.2 M sucrose, the specimens were postfixed in 1% buffered OsO_4, rinsed and dehydrated in alcohol and propylenoxide. Tissues were contrasted en bloc with uranyl acetate saturated in 70% alcohol overnight and embedded in Araldite (CIBA). Semithin and ultrathin sections were cut on an Reichert OMU 3 ultramicrotome and stained with toluidine blue and lead citrate, respectively. The ultrathin sections were observed in a Siemens Elmiskop 102 electron microscope. Fig. 1 shows the experimental arrangement and the sites of morphological investigation in the goldfish visual system.

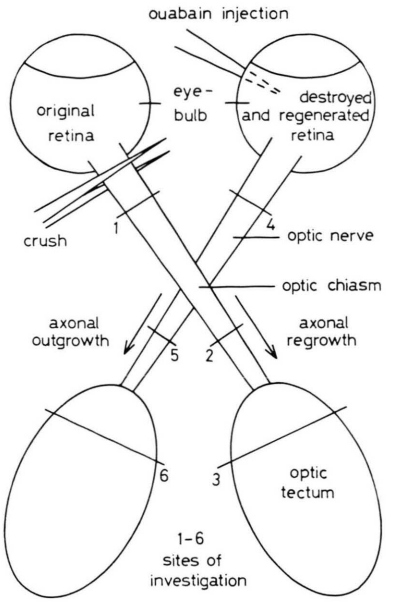

Fig. 1. Schematic representation of the experimental arrangement. The numbers are correlated to the sites of morphological investigation. After ouabain injection the retina is damaged and a new one is developed. The new ganglion cells send their axons into the degenerating visual system. On the contralateral side the optic nerve is crushed; since there is no retrograde retina degeneration, the proximal stumps of the original retina ganglion cell axons grow into the optic nerve and tectum. In the report are described the different modes of fiber regeneration and myelination.

Results

Regeneration of the optic fibers after nerve crush

The first regenerating fibers are seen 8 days after crush in the optic nerve about 2 mm distal from the damage zone (1 in Fig. 1; Fig. 2). They lie in groups in direct proximity to astrocytes and oligodendrocytes, measuring about 0.2 – 1.0 μm in diameter and containing a few microtubules and microfilaments and sometimes a mitochondrion. In the region behind the optic chiasm (2 in Fig. 1) there are no regenerating fibers, only degenerated debris from axons and myelin sheaths are seen here. In the following time the regenerating fiber bundles near the eye-bulb, which increase steadily in number, are embraced by astrocytic processes (Fig. 3), as demonstrated by Peters and Vaughn [20] in the rat optic nerve during ontogenetic development. The degenerating debris is removed by astrocytic myelophages and mesenchymal macrophages, which invade the nerve via the endoneurium. Glial cells are increased in number, and also the oligodendroglia participates in phagocytosis of degenerated material (Fig. 4). Phagocytosis by oligodendroglia was described in kitten [21], monkey [22] and man [23]. In spite of glial proliferation and intense process branching the glial scar does not prevent the fiber sprouting.

16 days after crush, no fiber in the proximal parts of the optic nerve near the eye-bulb (1 in Fig. 1) is myelinated (Fig. 3). Here, and in the segment behind the chiasm, the first myelinated fibers are scarcely seen 28 days after crush; after 38 days the myelogenesis is not strongly proceeded

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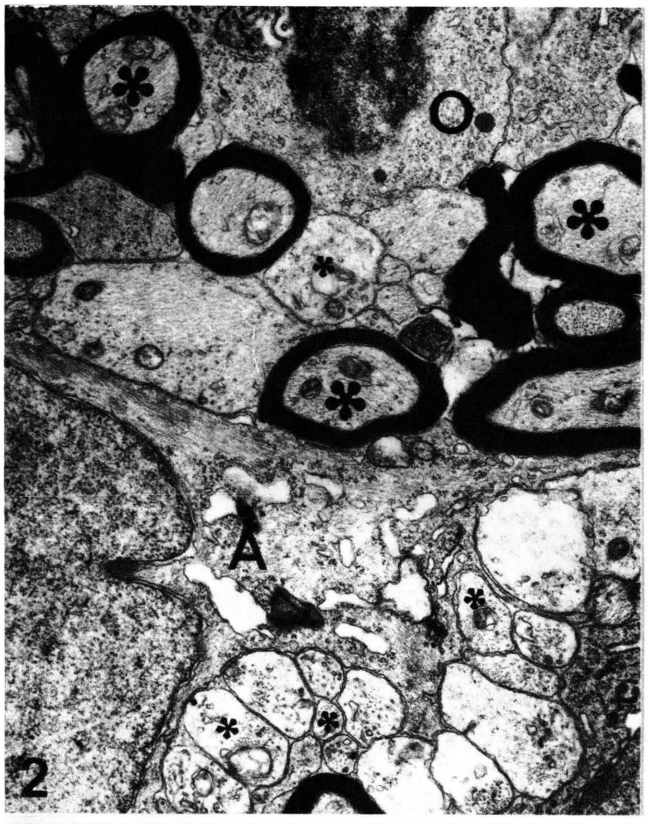
Fig. 2. Optic nerve 8 days after crush (1 in Fig. 1). Some fibers and myelin sheaths are still intact (large asterisks). The first axon regenerates (small asterisks) from the proximal stump have arrived the distal stump. They lie direct contact of astrocytes (A) and oligodendrocytes (O); 12 000 : 1.

Fig. 3. Optic nerve 16 days after crush (1 in Fig. 1). Astrocytic (A) and oligodendrocytic (O) processes embrace groups of unmyelinated axons. Macrophages (M) are present removing degenerated debris from the nerve; 6000 : 1.

Fig. 4. Optic tectum 16 days after crush (3 in Fig. 1); the same animal as in Fig. 3. Axon regenerates have arrived the tectum, the first fibers are remyelinated (asterisks) by phagocytosing oligodendrocytes (O); 6000 : 1.

Fig. 5. Optic tectum 16 days after crush (3 in Fig. 1); the same animal as in Figs 3 and 4. Synaptogenesis in the ventral optic layer is strongly in progress; 16 000 : 1.



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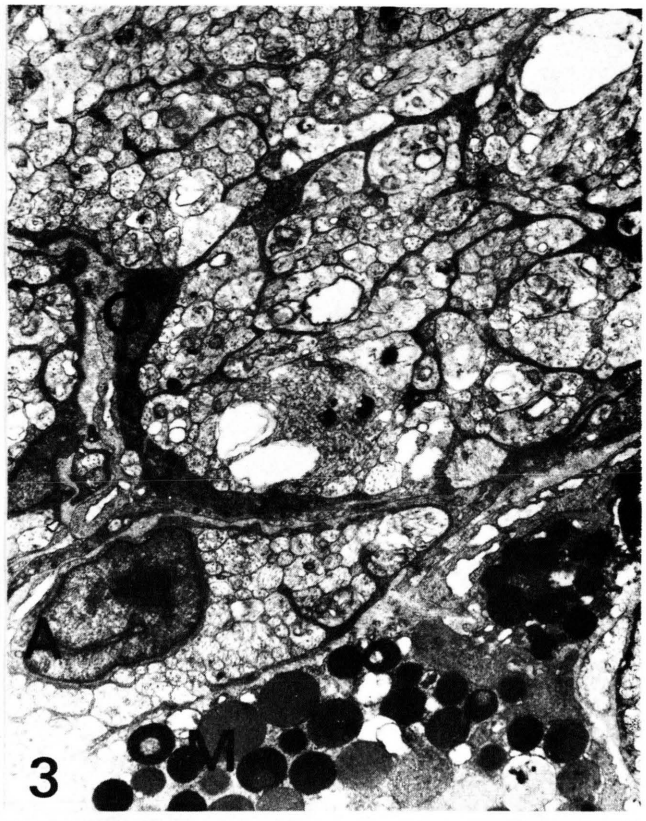

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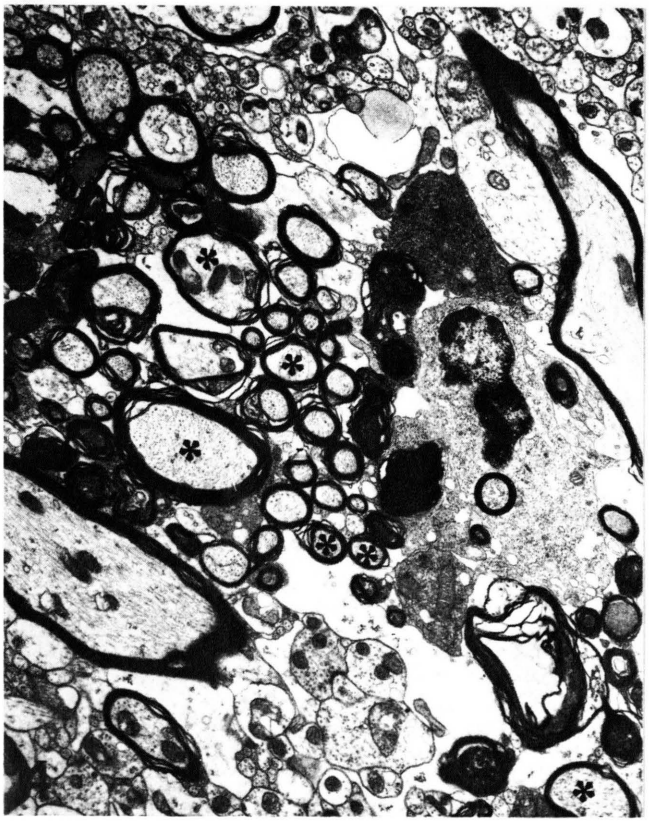

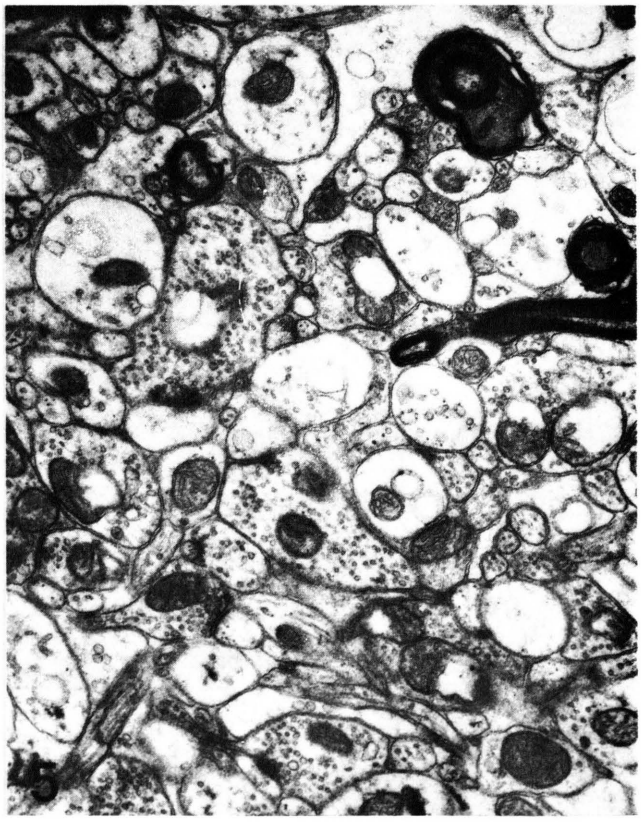


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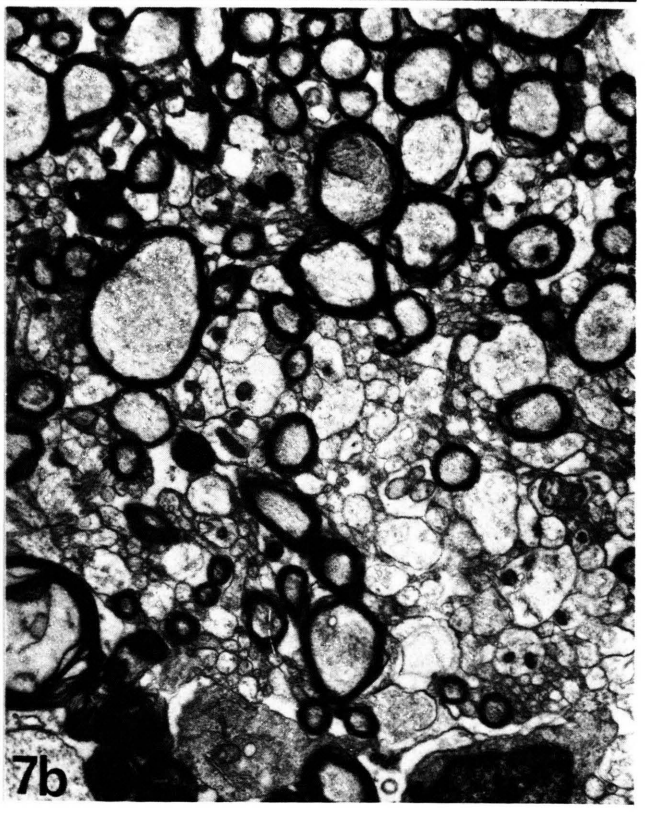
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(Fig. 6 a). However, after 57 days more fibers in the distal parts of the optic tract (2 in Fig. 1) are myelinated than in the proximal region (1 in Fig. 1).

In the optic tectum (3 in Fig. 1) the fibers of the optic layer including the synaptic endings, are degenerated. This degeneration of the synaptic endings is not of the dark type; they are swollen, the number of the synaptic vesicles is reduced, the synaptic mitochondria are damaged. Also the postsynaptic compartment is swollen, but still in contact with the degenerated presynaptic ending.

In the tectum regenerating fibers can be seen at first 16 days after the crush arranged in groups of 100 – 130 between degenerating material and large myelinated fibers, which have persisted longer than others (Fig. 4) (for comparison see Stensaas and Feringa [6]). Among the bundles of unmyelinated axon regenerates one can find both single newly myelinated fibers of different diameter, which are in contact with oligodendrocytes (Fig. 4), and newly developed synaptic formations. These are recognizable by their greater electron density of synaptoplasm, the larger number of synaptic vesicles and the existence of presynaptic microtubules (Fig. 5). Before the reestablishment of these new synapses remyelination of axon terminals is never found. 38 and 57 days after crush the number of both myelinated fibers and oligodendroglial cells in the tectum has enlarged (Fig. 7 a). For conclusion, one can say that the myelination process begins together with or shortly after synaptogenesis and earlier in the tectum that in the optic nerve, furnishing proof that it proceeds in retrograde direction.

73 and 85 days after crush most fibers in the optic nerve are myelinated, but clearly below the normal extent (Fig. 8 a). In the tectum, there are still many unmyelinated fibers of small diameter (Fig. 9 a). It is supposed, that one part of these fibers are branches of the terminal arborization of optic fibers, which do not succeed in establishing synaptic contacts and may remain unmyelinated. The other part of unmyelinated fibers may belong to neurons, whose axons remain completely unmyelinated. There is no means to distinguish clearly between both groups of fibers.

Regeneration of optic fibers following retina regeneration

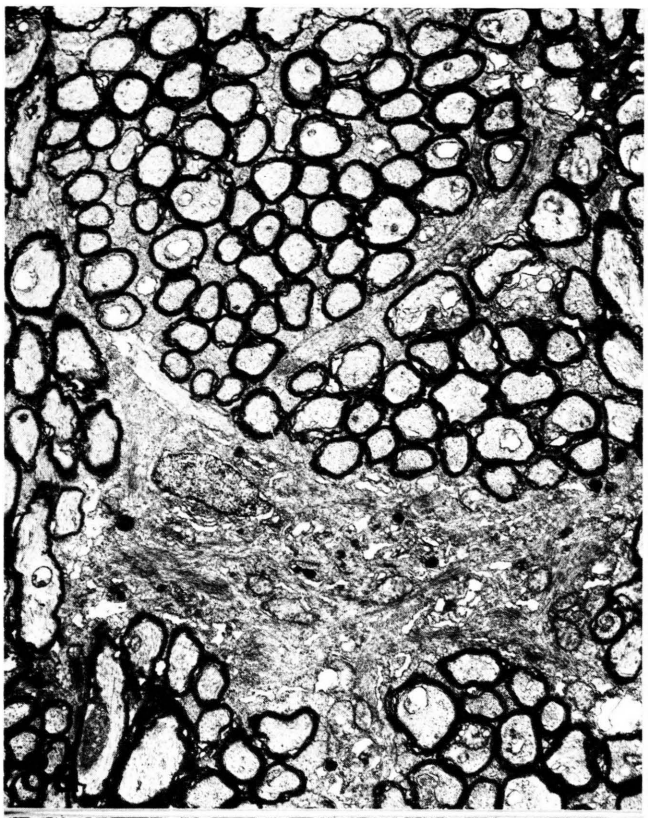
The delay of appearance of axonal sprouts in the tectum after ouabain-induced retina destruction and formation of a new retina is obviously no greater than the delay of regrowing of axons after crush. In any case, 16 days after ouabain injection the first regenerating fibers near the eye-bulb (4 in Fig. 1) can be found between degenerated ones and stimulated glial cells in the nerve, and also in the tectum (6 in Fig. 1) larger fiber bundles, probably resulting from terminal branching, have arrived. The first synapses are established, and the proliferated oligodendroglial cells begin the myelination. After 28 days, first myelination processes are seen within the optic nerve near the eyebulb, as similarly observed in the contralateral nerve after crush. Now only a few fibers are myelinated, they seem to be completely embraced by astrocytes, in some cases extremely thin processes of oligodendroglial cells are seen connecting the fiber with the glial cell body. This situation is nearly unchanged still 38 days after ouabain injection (Fig. 6 b). At this time, in the segment behind the chiasm (5 in Fig. 1) the myelogenesis has clearly advanced to a more developed stage. In the optic tectum there are both many myelinated and very small unmyelinated fibers (Fig. 7 b); the synaptic fields are fully developed. 57 days after injection, there has occurred a high rate of myelination. The optic layer in the tectum now appears nearly undistinguishable from the control, concerning both the myelinated fibers and the tectal morphology. In the optic nerve myelination is nearly completed, too, but some fascicles are retarded in remyelination, and in the endoneurium and the perineural sheath some macrophages give evidence from the former damage of the nerve. 73 and 85 days after ouabain injection the architecture of the visual system appears almost normal (Figs 8 b, 9 b).

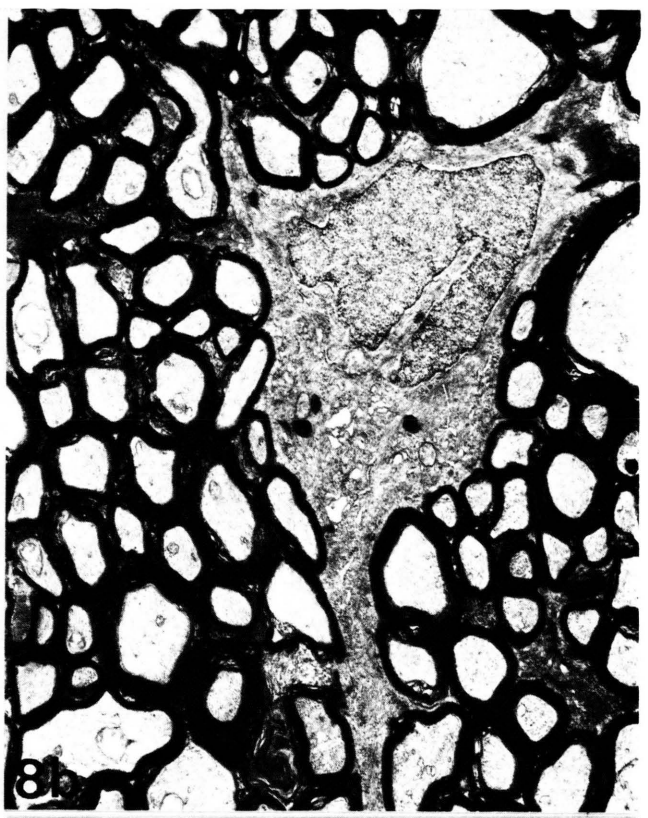
Fig. 6 a. Optic nerve 38 days after crush (1 in Fig. 1). Only a few fibers are slightly myelinated. Some unmyelinated fibers (large asterisks) show a larger diameter than some myelinated fibers (small asterisk); 20 000: 1.

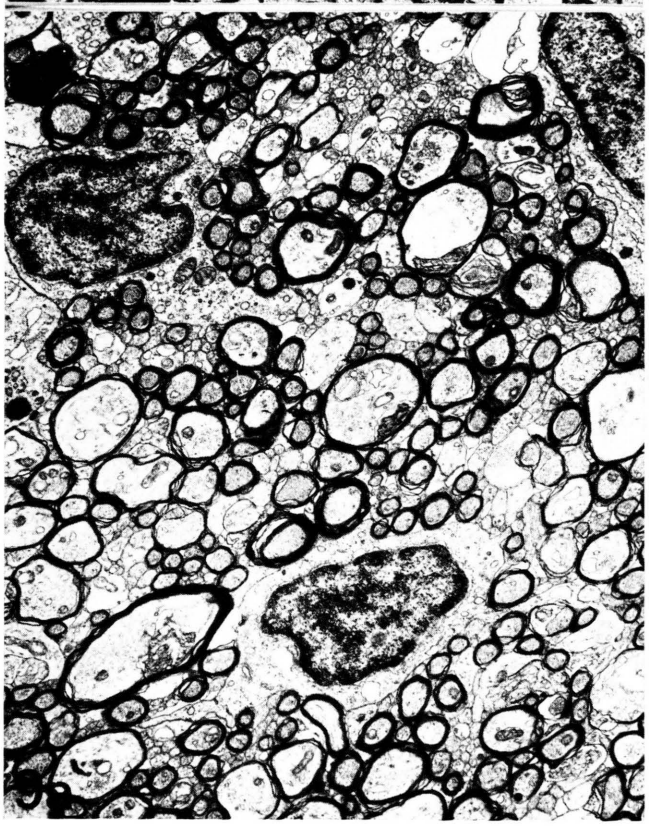
Fig. 6 b. Optic nerve 38 days after ouabain injection (4 in Fig. 1); the same animal as in Fig. 6 a. The myelination process is still at an early stage: 30 000: 1.

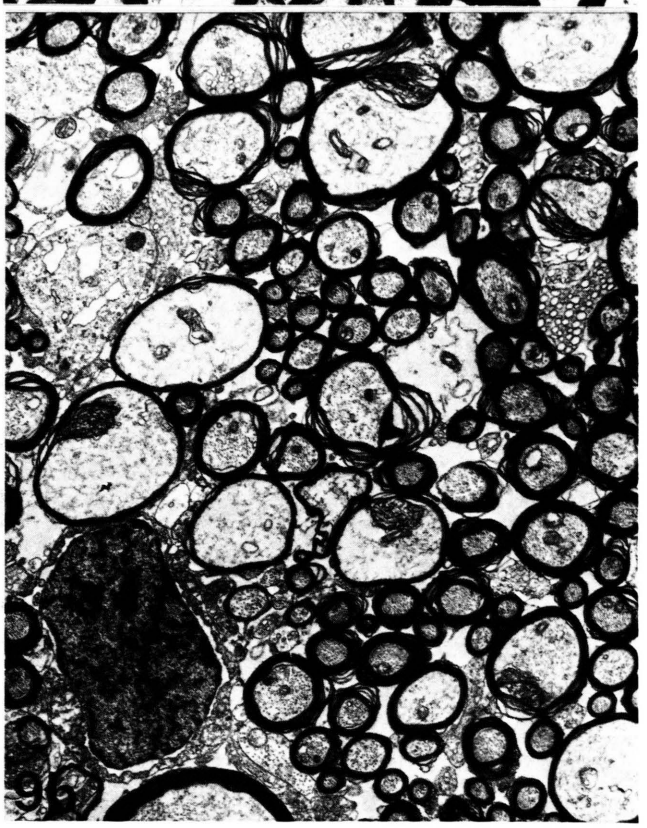
Fig. 7 a. Optic tectum 38 days after crush (3 in Fig. 1); the same animal as in Fig. 6. The oligodendroglia (O) is proliferated, synaptogenesis and remyelination are in progress: 6000: 1.

Fig. 7 b. Optic tectum 38 days after ouabain injection (6 in Fig. 1); the same animal as in Fig. 6. The extent in remyelination of axon regenerates is clearly higher than in the proximal parts of the nerve (Fig. 6 b); 6000: 1.









Compared with the contralateral side, where the nerve was crushed and the remyelination was incomplete, the nerve fibers projecting from the newly generated retina are remyelinated faster and stronger. But a lot of fibers remain unmyelinated; these can partly be seen also in the normal tectum, partly be explained in the regenerated optic layer as a surplus of arborization [5] and failure in establishing synaptic connections.

Discussion

The main results of this study are: 1. the remyelination of regenerated axons in the optic nerve of the goldfish is distinctly stronger with axons that have grown out from newly generated retina ganglion cells than after outgrowth of crushed fibers. 2. the remyelination process proceeds in retrograde direction; 3. regenerated axons are not remyelinated before synapses have developed.

It is generally assumed that myelogenesis starts after the glial cell has contacted the axon [24, 25]. In this report intimate connections between oligodendroglial cells and optic axons could be found long before the remyelination begins (Figs 2, 3). Preterminal axons are myelinated only if first synapses have developed in the same area (Fig. 4). In a study about ontogenetic structural changes in the trout optic tectum Rahmann and Jeserich [26] claim that "the end of the synaptogenetic period is characterized by the onset of myelination". The findings presented here, suggest that the onset of remyelination could be triggered by the synaptogenesis. This is in accord with the statement of Murray [5] that after "the establishment of some synaptic contact ... remyelination begins". Karlsson [27] found in the lateral geniculate nucleus of the postnatal rat a relationship of synaptogenesis to the onset of myelination after opening of the eyelids. In other investigations about myelination the relationship to synaptogenesis is not considered [4, 28 – 35]. Nevertheless, Rager [31] found at the 15. – 16. day of incubation in the chick optic nerve the begin of myelination, and in the following study [32] first synapses in the tectum are described at the 11. day of incubation. Reier and Webster [30] observed 35 days after crush of the *Xenopus* tadpole optic nerve a remyelination; a foregoing synaptogenesis could be assumed, but was not demonstrated.

If there is a causal relationship between synaptogenesis and myelination, the onset of myelin formation near the axonal terminals would be plausible as like as a failure in myelin formation in such fibers, which are not successful in establishing synaptic contacts. In the present study myelinating oligodendrocytes could be observed at first near the newly established synapses (16 days after injection or crush), whereas about 7 – 8 mm more proximal in the optic nerve comparable myelination stages appeared only about 20 days later (compare Figs 4 and 6 a). The finding of this retrograde remyelination is in accordance with the disto-proximal decrease of the cholesterol-content in human optic nerve during myelin development [28, 29] and the centripetal gradients in myelination found in the visual system of the rabbit [36] and the rat [37]. The contradictory results of Attardi and Sperry [1] in goldfish, Moore *et al.* [38] in cat and Skoff [35] in rat optic tract or nerve can not be explained. In any case, the attaining of a critical fiber diameter as an inductor of myelogenesis, accepted by many authors (for example [39 – 42]) and contradicted by others ([33, 38]; see Fig. 6 a) may not be the only mechanism that triggers myelination. Synaptic contact may likewise have an inducing influence on myelination, perhaps by means of establishing the fiber diameter necessarily. Further, large bundles of unmyelinated fibers in the terminal region both after nerve crush and retina regeneration are believed to be excess arborizations of regenerated fibers. Murray [5] has demonstrated an intense branching of regenerating axons in the goldfish optic tectum

◀ Fig. 8 a. Optic nerve 85 days after crush (1 in Fig. 1). All debris is removed, the architecture of the nerve is normalized, but the myelination is still below the normal extent; 6000 : 1.

Fig. 8 b. Optic nerve 85 days after ouabain injection (4 in Fig. 1). The same animal as in Fig. 8 a. The structure of the optic nerve is widely not to distinguish from a control. The axons are of larger caliber and the remyelination is stronger than in the contralateral nerve crushed 85 days before; 6000 : 1.

Fig. 9 a. Optic tectum 85 days after crush (3 in Fig. 1); the same animal as in Fig. 8. The myelination is not so uniform as in the nerve (Fig. 8 a); most fibers are myelinated below the normal extent or unmyelinated. The number of glial cells is still enlarged in comparison with the control; 6000 : 1.

Fig. 9 b. Optic tectum 85 days after ouabain injection (6 in Fig. 1); the same animal as in Fig. 8. The myelination is stronger than in the contralateral tectum (Fig. 9 a). A relative small number of large fibers are myelinated below the normal extent; 6000 : 1.

28 days after section of the optic tract. These widely branched arborizations are assumed to be reduced to a distinct projective field [43]. It could be speculated that these branches fail to establish synaptic contact and are therefore not myelinated, enlarging the number of unmyelinated fibers in the tectum in comparison to the unmyelinated fibers in the optic nerve.

Axons of newly developed ganglion cells are more completely ensheated by oligodendrocytes in the same time space than axons which had grown out from original ganglion cells after crush (compare Figs 8 a, 8 b, and 9 a, 9 b, respectively). This observation could reflect a problem of cellular age: a young axon is more potent to induce the myelination process in oligodendroglial cells than an older axon. In both modes of regeneration the oligodendroglia has proliferated; this excludes the possibility that in the glia itself is localized an age-dependent trigger mechanism regulating the myelogenesis. Studies about myelination of the optic nerve in ontogenesis do not seem to exist for the goldfish, but from other species (chick: Rager [31], trout: Rahmann and Jeserich [26]) it can be concluded, that during ontogenesis the optic nerve development and myelination proceeds continously and occurs in a similar manner and velocity as shown here after ouabain induced retina degeneration and following regeneration.

This means that there is a dependence between the age of the neuron and its potency to influence the myelination of its axon by the glial cell. It has been shown that an axonal factor induces and regulates the myelination in the glial cell in the peripheral nervous system [44–47]; in the visual system a dependence of the myelination of the optic fibers on visual function was shown by Gyllensten and Malmfors [48].

The nature of this axonal factor characterized by its triggering effect upon glial cells to myelinate the axon, its dependence on the establishment of synaptic contact, its extension in a retrograde direction along the axon and by its stronger effect in young than in older axons remains to be shown by future work.

This work was supported by grant Wo 215/4 from the Deutsche Forschungsgemeinschaft. I am gratefully indebted to Dr. G. Kurz-Isler for expert help in electron microscopy. Prof. Dr. W. Schlote is thanked for critical reading the manuscript and Mrs. B. Sabrowski for typing it and designing Fig. 1.

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