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Myelination and Remyelination in the Regenerating Visual System of the Goldfish*

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Summary. The remyelination of regenerated optic axons was investigated in goldfish following either optic nerve crush or ouabain retinal intoxication. Axons grown after nerve crushing acquire thinner myelin sheaths than axons originating from reconstituted ganglion cells. If axons of reconstituted ganglion cells are crushed and allowed to regenerate, the subsequent myelination is weaker than that of control axons not interrupted by crushing, but stronger than that of axons of preexisting retinal ganglion cells.

The present results suggest that a neuron is capable of inducing a normally developed myelin sheath when its axon contacts an oligodendrocyte the first time, whereas a neuron whose axon contacts an oligodendrocyte the second time is not capable of forming a normal myelin sheath in the adult animal. The present results also support the notion that the oligodendrocyte requires a neuronal signal for myelin sheath formation.

Key words: Remyelination – Axonal regeneration – Oligodendrocytes – Optic nerve – Goldfish

Myelination is the result of a special interaction between a neuron and a myelin-producing glial cell. The phenomenon consists of two distinguishable steps, the synthesis of myelin-specific components and the formation of a myelin sheath. In the peripheral nervous system both steps require the presence of neurons. Mirsky et al. (1980) have shown that Schwann cells stop synthesizing myelin-specific molecules after isolation from neurons, and according to Weinberg and Spencer (1975, 1976) and Aguayo et al. (1976a, b), the peripheral axon is able to induce myelination in the Schwann cell. In the central nervous system oligodendrocyte synthesis of myelin-specific compounds does not require a continuous signal from axons (Mirsky et al. 1980); however, the necessity of an axonal signal to initiate the formation of a myelin sheath has not yet been verified.

In the present study the myelination of regenerating ganglion cell axons in the visual system of the goldfish will be described. Differences in oligodendrocyte myelination of axons grown from reconstituted ganglion cells after retinal intoxication are interpreted as the expression of an axonal mechanism which triggers myelination activity in the oligodendrocytes of the optic nerve.

Materials and Methods

The optic nerve of goldfish (Carassius auratus) was used in the present experiments designed to study myelination and remyelination of regenerating optic axons.

In a first series of experiments, the left optic nerve of 8 animals was crushed behind the eyebulb for 2–4 s using watchmaker's forceps. On the right side, ouabain (Serva; 5 or 10 µl 10^{-5} M) was injected intraocularly. During surgery the animals were anesthetized with 0.02% MS 222 (Sandoz). The body length of the fish was between 10 and 12 cm; the final concentration of intraocularly injected ouabain was approximately 1.5 to $3 \cdot 10^{-6}$ M, varying slightly according to the respective eyebulb volume (Easter et al. 1977; Johns and Easter 1977). After an experimental time of 35–85 days the animals were sacrificed.

In another series of experiments, the right eye of nine animals was injected with ouabain and the retina plus optic nerve were allowed to regenerate for 30 or 60 days (first regeneration period). Both optic nerves were then crushed behind the eyebulb and allowed to regenerate for another 30–100 days (second regeneration period). In two animals, the left optic nerve was crushed simultaneously with the right ouabain eye injection and then a second time together with the right optic nerve after the first regeneration period.

In a third experimental group, the right eye of four animals was injected with ouabain and the retina plus optic nerve were

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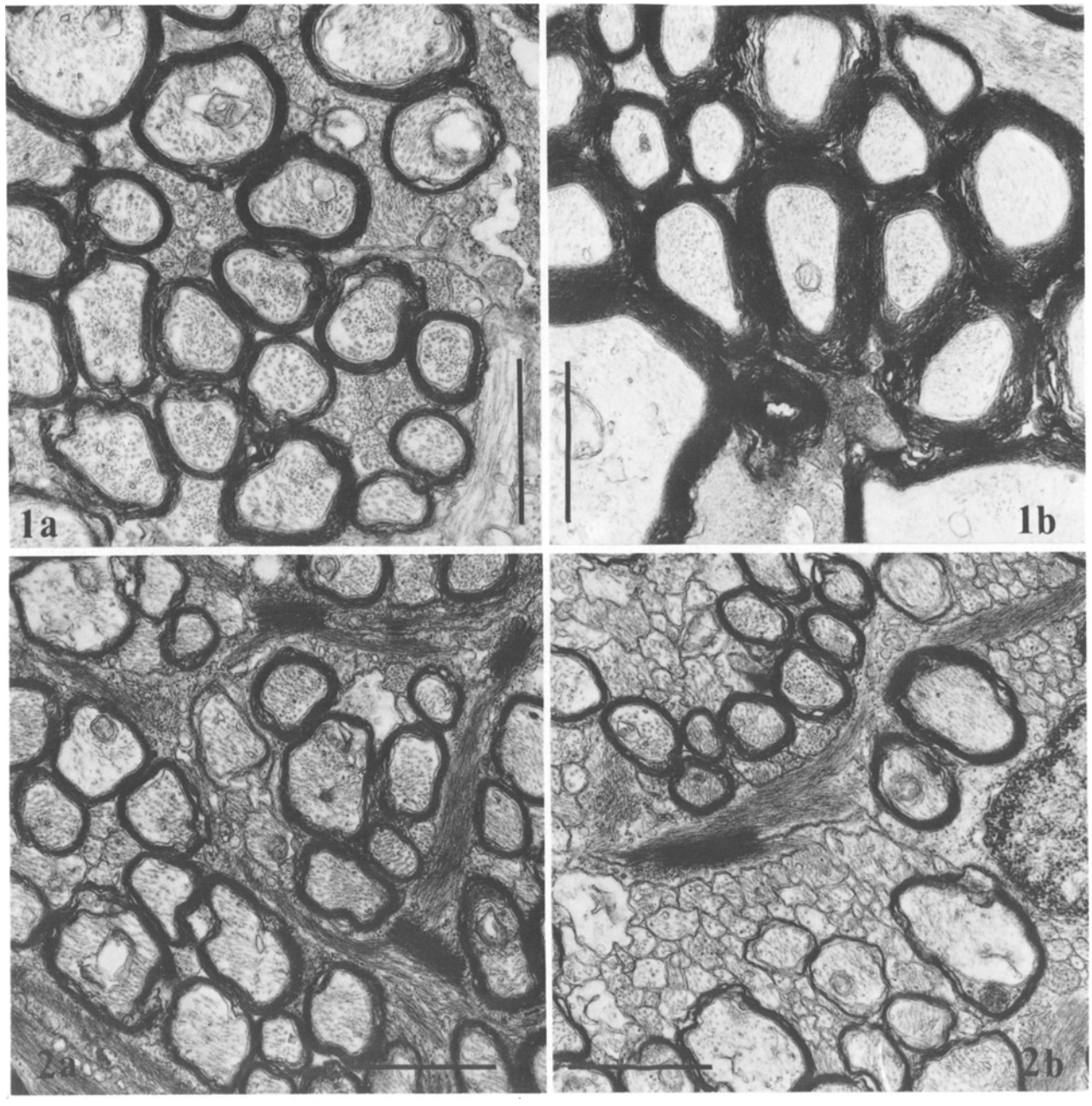

Fig. 1a, b. Goldfish optic nerves, 85 days after operation. The bars indicate 1 μm. × 25,000. **a** Left optic nerve, 85 days after retrobulbar crush. The myelination index is 0.82 ± 0.047. **b** Right optic nerve, 85 days after retinal intoxication by ouabain. The myelination index is 0.67 ± 0.074

Fig. 2a, b. Goldfish optic nerves, 160 days after the start of the experiment. The bars indicate 1 μm. × 23,000. **a** Left optic nerve, 60 days after retrobulbar crush. The myelination index is 0.83 ± 0.052. Axon bundles are embraced by astrocytic processes connected to each other by desmosomes. **b** Right optic nerve, 160 days after retinal intoxication by ouabain and 60 days after retrobulbar crush of the regenerated optic nerve. The myelination index is 0.79 ± 0.068. Between the myelinated axons there is a large number of tiny unmyelinated axonal profiles which are absent in this quantity in the contralateral optic nerve

allowed to regenerate for 30 days (first regeneration period). Thereafter, the right optic nerve was crushed behind the eyebulb simultaneously with a left ouabain eye injection. The subsequent second regeneration period took 80 or 100 days.

After an experiment was ended by decapitation of the animal, the retina, optic nerve and optic tectum were removed and fixed by immersion in 2% glutaraldehyde (pH 7.4) buffered with 0.1 M cacodylate. After rinsing in cacodylate buffer containing 0.2 M sucrose, the tissue was postfixed in 1% buffered OsO_4 and dehydrated in ethanol. Subsequently, the tissue was stained en

Table 1. Morphometric analysis of the myelin sheath thickness of some selected representative optic nerves. In the left column the experiment is described. a.c. – after crushing, a.o.i. – after ouabain injection, n – number of measured axons per optic nerve, d – axon diameter, D – fiber diameter, SD – standard deviation. The significance of differences was checked with Student's *t*-test. Remarkably, myelin sheath thickness expressed as the ratio of axon to fiber diameter cannot be correlated directly with the axon diameter itself. In most experiments smaller axons are relatively more thickly myelinated than larger axons. This observation contradicts the major role of axon diameter in affecting myelin sheath thickness and corroborates a direct metabolic influence of the neuron on the myelinating activity in the oligodendrocyte

Experiment	d (μm) SD	*t*-test	D (μm) SD	*t*-test	$\frac{d}{D}$ SD	*t*-test
I.						
85 days a.c.						
(left side)						
n = 412	0.73 ± 0.36		0.87 ± 0.23		0.82 ± 0.047	
85 days a.o.i.						
(right side)		n.s.		$P < 0.001$		$P < 0.001$
n = 340	0.77 ± 0.67		1.07 ± 0.36		0.67 ± 0.074	
II.						
78 days a.c.						
(left side)						
n = 374	0.62 ± 0.19		0.74 ± 0.21		0.84 ± 0.049	
108 days a.o.i., 78 days a.c.						
(right side)		$P < 0.001$		$P < 0.001$		$P < 0.001$
n = 372	0.45 ± 0.19		0.58 ± 0.21		0.77 ± 0.074	
III.						
115 days a.c., 55 days a.c.						
(left side)						
n = 360	0.64 ± 0.27		0.77 ± 0.29		0.83 ± 0.056	
115 days a.o.i., 55 days a.c.						
(right side)		$P < 0.001$		$P < 0.001$		$P < 0.001$
n = 323	0.46 ± 0.16		0.60 ± 0.42		0.79 ± 0.065	
IV.						
60 days a.c.						
(left side)						
n = 355	0.59 ± 0.24		0.71 ± 0.25		0.83 ± 0.052	
160 days a.o.i., 60 days a.c.						
(right side)		$P < 0.001$		$P < 0.001$		$P < 0.001$
n = 340	0.48 ± 0.21		0.60 ± 0.23		0.79 ± 0.068	
V.						
100 days a.c.						
(left side)						
n = 400	0.63 ± 0.28		0.77 ± 0.32		0.81 ± 0.066	
130 days a.o.i., 100 days a.c.						
(right side)		$P < 0.05$		n.s.		$P < 0.001$
n = 365	0.58 ± 0.35		0.74 ± 0.37		0.77 ± 0.063	
VI.						
110 days a.o.i., 80 days a.c.						
(right side)						
n = 326	0.50 ± 0.19		0.64 ± 0.22		0.77 ± 0.069	
80 days a.o.i.						
(left side)		$P < 0.001$		$P < 0.001$		$P < 0.001$
n = 344	0.67 ± 0.25		0.91 ± 0.31		0.72 ± 0.065	

bloc overnight in 70% ethanol saturated with uranylacetate. Dehydration was continued thereafter using absolute ethanol and propylenoxide. The tissue was then embedded in Araldite (Ciba) and sectioned on a Reichert OMU 3 ultramicrotome. Semithin sections were stained with toluidine blue, ultrathin sections with lead citrate. The ultrathin sections were examined in a Siemens Elmiskop 102 or Zeiss EM 10 C electron microscope.

Representative cases in the experimental material were selected for morphometric analysis of myelin sheath thickness. The axonal diameter (d) and fiber diameter (D) of more than 300 myelinated fibers in each selected optic nerve were measured at a final magnification of 30,000. The ratio of axonal diameter to fiber diameter ($\frac{d}{D}$) was used to calculate the degree of axonal myelination (myelination index).

Results

After retrobulbar crush of the optic nerve, there was an interval of 4–6 days before the first appearance of axonal regenerates in the distal stump. The axons grew in bundles, which were embraced by processes of astrocytes and oligodendrocytes. Although there was very close contact between oligodendrocytes and axons for many days, the first myelin sheaths were only developed in the optic nerve about 30–40 days after crushing, i.e. when the axons reached the tectum, established synaptic contacts and acquired distal myelin sheaths. In the course of axonal regeneration, myelin sheaths always appeared earlier in the tectum than in the optic nerve (Wolburg 1978, 1981). While the first myelinated fibers were dispersed irregularly throughout the optic nerve, the number of myelinated fibers increased noticeably (Fig. 2a), and finally became normal about 70–90 days after crush (Fig. 1a). The myelination index in the regenerated optic nerve after retrobulbar crush exceeded 0.80 (Table 1, experiments I, II, IV, V, left side) whereas the index of optic nerve fibers in normal animals was in the order of 0.65–0.77 (material not shown here).

In another experiment, ouabain was injected into the eyebulb of the same animals on the side contralateral to the retrobulbar optic nerve crush. A certain threshold concentration was found to be within the range of intraocular ouabain concentrations. Below the threshold, the retina was slightly damaged with necrosis of ganglion cells and of some cell bodies of the inner nuclear layer. Above the threshold concentration the entire, differentiated retina was damaged. The threshold concentration was in the order of $2 \cdot 10^{-6}$ to $5 \cdot 10^{-6}$ M ouabain (Wolburg, in prep.). When totally damaged, the retina was able to regenerate slowly from the marginal growth zone (Maier and Wolburg 1979). When slightly damaged, the retina could regenerate quickly from both the marginal growth zone and the outer nuclear layer (Maier and Wolburg 1979). The axons of the reconstituted ganglion cells grew nearly synchronously with, or a few days after, the axons of the contralateral side after optic nerve crush (Wolburg 1981). This was observed approximately 30 days after crushing or retinal intoxication when myelination of the regenerated axons of both optic nerves was very poor and of a minimal percentage. In contrast, when damage to the retina was more severe due to a dose just above threshold, regeneration was lesser in the early stage in comparison to the side with optic nerve crush (material not shown here). Consequently, the myelinating activity in both regeneration modes can only be compared using visual systems in which regeneration velocities are nearly the same. Regeneration velocity should be checked by examining the condition of both the regenerated retina and the regenerated optic nerve fibers of both systems.

Such a comparison of both regenerating optic nerves revealed that regeneration and myelination in both systems was nearly equal 40–50 days after the start of a subthreshold experiment. Later, however, myelin thickness of the axons originating from regenerated retina (cf. Fig. 4 left) increasingly exceeded that of the axons regenerated from the proximal stumps after crushing (cf. Fig. 4 right). About 60–85 days after the beginning of an experiment, the axons which had grown from preexisting ganglion cells after optic nerve crush showed myelin sheaths that were thinner than the axons of the contralateral side which had grown from newly established ganglion cells (Fig. 1a, b; for quantitative results see Table 1, experiment I).

To investigate regeneration and myelination of axons from preexisting (cf. Fig. 4 right) and young neurons (cf. Fig. 4 left) under equivalent experimental conditions, both optic nerves had to be crushed. Both of them, the regenerated one and the preexisting one, were crushed behind the eyebulb at different times after retinal intoxication (see Materials and Methods) when equivalent retinal and optic nerve regeneration could be expected on the basis of earlier experiments. In some other animals, the left optic nerve was crushed simultaneously with contralateral retinal intoxication, and after the regeneration period, both nerves were crushed, the left one a second time, the right for the first time. After the second regeneration period, myelination of the axons was measured.

The axons which had grown from a reconstituted retina and thereafter from the proximal stump after crushing showed a myelination index in the order of 0.78 (Table 1, experiments II–V, right side; Figs. 2b, 3b; cf. Fig. 4 middle). The difference between this myelination index and the index in an optic nerve originating from a regenerated retina without subsequent interruption by crushing (cf. Fig. 4 left) was highly significant. Furthermore, the myelin sheaths of the former (cf. Fig. 4 middle) were thicker than those on the contralateral side, where the optic nerve derived from an untreated, preexisting retina was crushed once or even twice (Table 1, experiments II–V, left side; cf. Fig. 4 right). Consequently, three degrees of myelination could be distinguished (Fig. 4). After ganglion cell reconstitution, the axons showed a myelination index in the normal range. When the axons from a reconstituted ganglion cell were crushed, the myelination index of the regenerated axons became significantly larger. The lowest

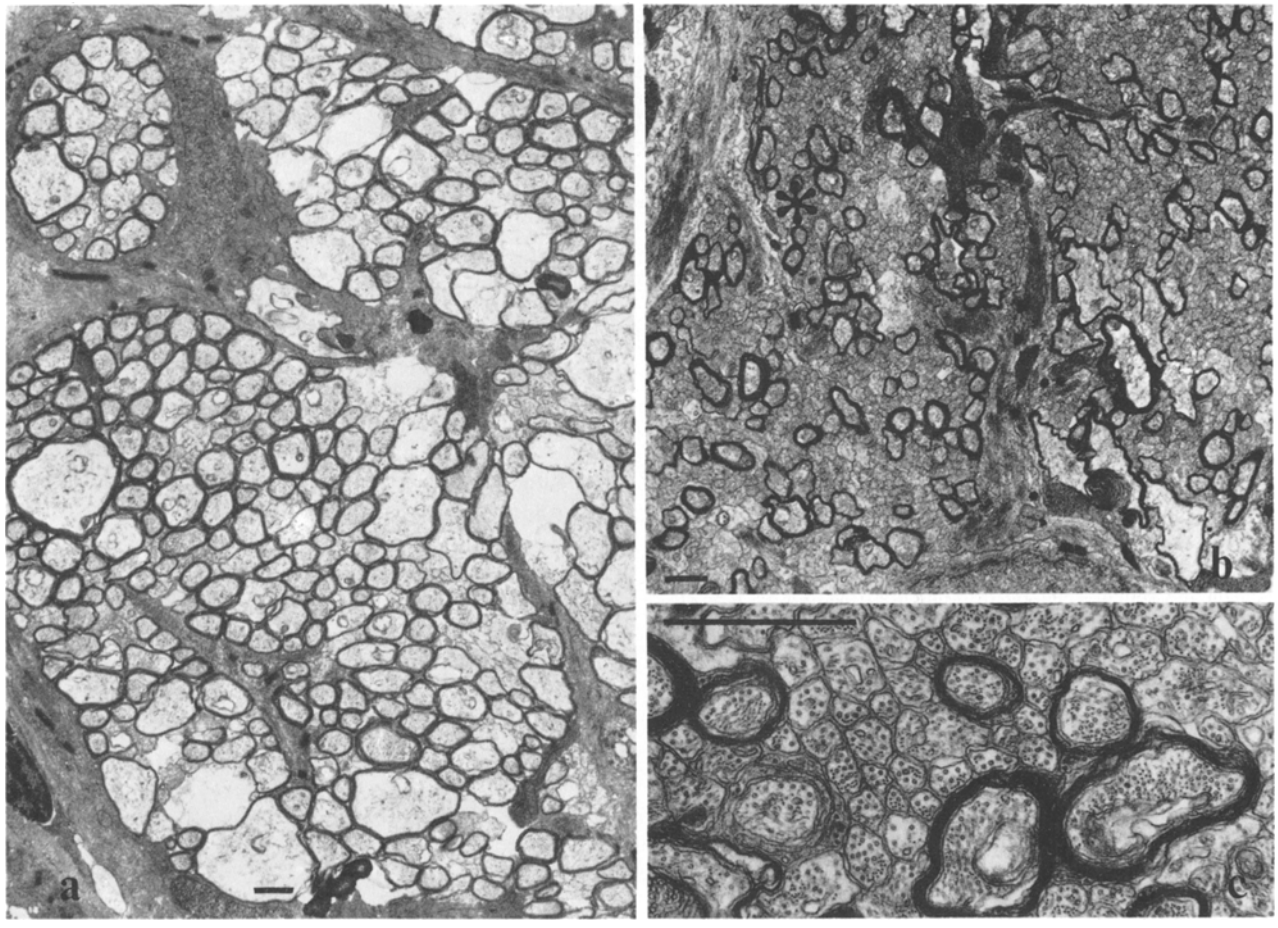

Fig. 3a–c. Goldfish optic nerves, 115 days after start of the experiment. × 4,800 (**a, b**); × 25,000 (**c**); the bars indicate 1 μm. **a** Left optic nerve, 115 days after the first and 55 days after the second retrobulbar crush. The myelination index is 0.83 ± 0.056. **b** Right optic nerve, 115 days after retinal intoxication by ouabain and 55 days after retrobulbar crush of the regenerated optic nerve. The myelination index is 0.79 ± 0.065. The asterisk indicates the region shown in higher magnification in **c. c** Detail from **b.** Very small axonal profiles are seen between the larger myelinated axonal regenerates. Such profiles are not observed in this quantity in the contralateral optic nerve and are therefore suggestive of intense axonal sprouting after crushing of the optic nerve belonging to a reconstituted retina

degree (highest index) of myelination was reached when axons derived from a preexisting retina were crushed and then regenerate.

In additional experiments the effect of crushing on myelination was reconfirmed by bilateral intoxication of the retina and unilateral crushing of the regenerated axons. The experiment was timed in such a manner that the axons of the regenerated retinal ganglion cells could grow simultaneously with the axons of the contralateral side after crushing. Myelination of crushed axons of young neurons was very significantly lower than that of uncrushed axons of young neurons (Table 1, experiment VI).

In all experiments in which axons from a newly developed retina were crushed and allowed to regenerate, a large number of tiny axonal processes, measuring sometimes as little as 0.1 μm in diameter, were subsequently observed (Figs. 2b, 3c). These processes were reminiscent of axonal sprouts and branches. Neither a regenerated optic nerve developed from a regenerated retina nor an optic nerve regenerated after crushing showed a comparable number of such tiny processes. The putative sprouting and branching phenomenon, which is yet to be definitely proved, suggests that axonal damage can stimulate this type of axonal growth to a higher degree in newly established than in preexistent neurons.

Discussion

The present study has shown an interrelationship between neurons and oligodendrocytes in myelination in the course of regeneration of the optic nerve of the goldfish. Two to three months after crushing

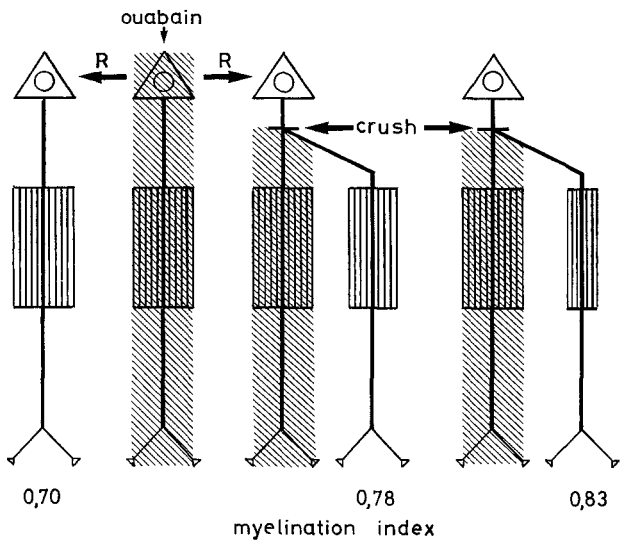

Fig. 4. Schematic summary of the results: after application of ouabain the retinal ganglion cells undergo necrobiosis and are reconstituted by mitotic processes (*R*). The axons of these reconstituted neurons are myelinated in the normal range (*left*). When crushed, the axons of reconstituted neurons regenerate and are thinner myelinated than is the case without crushing (*middle*), but thicker than axons of preexisting ganglion cells after crushing (*right*)

the optic nerve, the regrowing axons acquire a myelin sheath which remains thinner than the normal myelin sheath (Figs. 1a, 2a). When the retina is destroyed with ouabain the axons of the reconstituted retinal ganglion cells grow into the optic nerve and tectum, and within two to three months acquire a myelin sheath which seems to be in the normal range (Fig. 1b). Thus, although optic nerve crushing and retinal degeneration are both followed by Wallerian degeneration in the optic nerve, and accompanied by oligodendroglial and astroglial proliferation (Wolburg 1981), the myelinating activity is different in the two regenerating systems.

The term "myelinating activity" is used in the present paper in the sense that it is manifested morphologically by the thickness of the myelin sheath and the myelination index $\frac{d}{D}$. It is not possible, however, to distinguish morphologically between an altered rate of myelin synthesis and an altered rate of incorporation of presynthesized, myelin-specific glycolipids and proteins into the myelin sheath. With regard to metabolic disorder as a possible cause of hypomyelination, it should be noted that undernutrition has a negative effect upon myelination (Krigman and Hogan 1976; Reddy et al. 1979; Lai and Lewis 1980). In the present experiments, however, the optic nerve distal to the crush showed no morphological signs of nutritional deprivation, because the glial cells proliferated. Therefore, it seems likely that the axons degenerated exclusively because of their separation from the cell body, and the cause of the altered myelination is apparently localized in the glio-neuronal system itself. Furthermore, it must be noted that a relationship between axon diameter and myelin sheath thickness, which is a matter of dispute in the literature (for recent discussion see Lai and Lewis 1980), could not be corroborated by the present morphometric analysis (Table 1).

The present results are consistent, however, with the notion that a neuronal signal is required by the oligodendrocyte to initiate the formation of a myelin sheath. In principle, this signal could be initial impact cranking up the machinery for myelin formation in the oligodendrocyte; or it could be a continuous signal from the axon to the myelinating cell reflecting neuronal differences as differences in myelin sheath formation. In any case, contact with an axon appears to be a condition sine qua non for the formation of a myelin sheath (Bornstein and Murray 1958; Knobler et al. 1974).

With regard to the onset of myelin sheath formation and maintenance of that sheath, the nature of the axoglial contact is completely unknown. The experiments presented here suggest a metabolic basis for axoglial interaction: axons originating from a preexisting nerve stump induce less myelinating activity in the myelinating cells than axons from reconstituted neurons (Figs. 1a, b, 4).

It is conceivable that a neuronal factor influences the myelination activity of the oligodendrocytes. This factor may be activated by synaptogenesis, since the myelination process begins only after synaptogenesis has taken place (Murray 1976; Rahmann and Jeserich 1978; Wolburg 1981). The activated factor may be reversely effective retrogradely, which is consistent with the observation of distoproximal myelination (Friede and Hu 1967; Matheson 1971; Narang and Wisniewski 1977; Wolburg 1981). In principle, the factor could be provided by the postsynaptic cell in the optic tectum. Such a mechanism seems to be improbable, however, since crushing of the presynaptic optic axon could hardly have an effect on the myelinogenesis of the regenerated axon if the factor originated from the postsynaptic neuron. Thus, the hypothetical factor should originate from the presynaptic perikaryon, i.e., the retinal ganglion cell.

If the perikaryal substance does play a role in myelin stimulation, its synthesis cannot be stimulated by nerve crushing, in contrast to that of proteins and RNA (Grafstein and Murray 1969; Heacock and Agranoff 1976; Burrel et al. 1979). Indeed, crushing

results in decreased myelination activity in the regenerated axon (Figs. 1a, 2a, b, 3a, b), suggesting inhibition of either the synthesis, provision or transport initiation of the myelin stimulating factor, or a combination thereof. The crush-induced inhibition of the myelinating activity is most pronounced in fibers originating from preexisting retinal ganglion cells, and significantly weaker in fibers originating from reconstituted retinal ganglion cells.

It is essential to note that remyelination in the central as well as in the peripheral nervous system of an adult animal is always hypomyelination. This applies both to axons remyelinated either after regeneration (Murray 1976, for the CNS; Schröder 1972, for the PNS) or after demyelination without axonal damage (Bunge et al. 1961; Gledhill et al. 1973; Blakemore 1973, 1974; Ludwin 1978, for the CNS; Raine et al. 1969, for the PNS; for review about demyelination and remyelination in the PNS, see Allt 1976). However, Reier and Webster (1974) found a reorganization of myelin sheaths that were usually of normal thickness in a study of regeneration and remyelination of Xenopus tadpole optic nerve fibers.

Altogether, the above observations corroborate the notion that an adult animal axon – both of reconstituted and of a preexisting neuron – is not capable of completely reinducing the myelin formation in the myelinating cell, whereas at early developmental stages an axon can completely reinduce myelin sheath formation.

At the moment one can only speculate as to whether the observed decrease in signal intensity subsequent to the initial induction of myelination in adult animals is a result of reduced availability of the axonal factor, or an altered micro-environment of the neuron in the course of development, or whether the disturbance between the neuron and the myelinating cell is confined to the micro-architecture of certain constituents in the neuronal membrane.

Concerning the nature of the signal, Spencer and Weinberg (1978) have postulated that an enzymatically induced alteration of the axonal membrane is answered by a complementary alteration of the membrane of the myelinating cell. Ellisman (1979) has investigated the distribution of particles on the axolemma of myelinated and amyelinated fibers. The latter author suggests that the location of the particle patches defines the nodal region, signaling the Schwann cells not to form a myelin sheath there, and that the myelin sheath is therefore developed only between the patches. In another approach, Raine (1978) and Soffer and Raine (1980) have described various forms of axoglial specialization in the demyelinated CNS. Thus, further research should be directed toward characterizing membranes of regenerating or formerly demyelinated axons and comparing them to axons from reconstituted or ontogenetically very young nerve cell bodies.

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