Advances in Anatomy
Embryology and Cell Biology
67

Hartwig Wolburg

Axonal Transport, Degeneration, and Regeneration in the Visual System of the Goldfish

Springer-Verlag Berlin Heidelberg New York

Hartwig Wolburg

Axonal Transport, Degeneration, and Regeneration in the Visual System of the Goldfish

With 28 Figures

Springer-Verlag
Berlin Heidelberg New York 1981

Priv.-Doz. Dr. Hartwig Wolburg
Pathologisches Institut der Universität
Abt. für submikroskopische Pathologie
und Neuropathologie
Liebermeisterstr. 8
D-7400 Tübingen

ISBN 3-540-10336-8 Springer-Verlag Berlin Heidelberg New York
ISBN 0-387-10336-8 Springer-Verlag New York Heidelberg Berlin

Library of Congress Cataloging in Publication Data. Wolburg, Hartwig, 1945 – Axonal transport, degeneration, and regeneration in the visual system of the goldfish. (Advances in anatomy, embryology, and cell biology; v. 67) Bibliography: p. Includes index. 1. Visual pathways. 2. Axonal transport. 3. Nervous system - - Degeneration. 4. Nervous system - - Regeneration. 5. Goldfish - - Physiology. I. Title. II. Series. QL801.E67 vol. 67 [QP475] 574.4s [597'.01'823] ISBN 0-387-10336-8 (U.S.) 80-22731

Printed in Germany.

Composition: Schreibsatz Service Weihrauch, Würzburg
Printing and binding: H. Stürtz AG, Universitätsdruckerei, Würzburg
2121/3321-543210

Advances in Anatomy Embryology and Cell Biology

Vol. 67

To Karen and Thomas

Acknowledgments

This study was funded by the Deutsche Forschungsgemeinschaft. I am indebted to Prof. Dr. W. Schlote for helpful advice and numerous discussions. I am also grateful to Dr. G. Kurz-Isler for her generous help in problems dealing with electron microscopy and to Mrs. B. Sabrowski for her careful preparation of the manuscript. The careful translation of T.C. Telger is gratefully acknowledged. The translation was financially supported by the Erwin Riesch Foundation.

Contents

1 Introduction

One of the basic principles underlying the efficiency and adaptability of cellular metabolism is the structural compartmentalization of the cell. Only through compartmentalization can reaction components be kept apart prior to their reaction, isolated from other "reaction spaces" during the course of their reaction, and the reaction products incorporated into designated structures or transported to remote parts of the cell. Thus, the partitioning of the cellular substance into countless membranous spaces corresponds to the spatial segregation of reaction components, and the dynamics of intracellular membrane systems is an expression of ever-changing equilibrium conditions and the continuous formation of new reaction spaces. It has been shown with some certainty that many of the processes in membrane dynamics can take place only with the aid of contractile proteins such as actin, myosin, and tubulin.

These statements apply not only to spherical, cubic, prismatic, and polygonal cells, but also to the cell bodies of neurons. However, the nerve cell is distinguished from other cells mainly by its extreme polarity, which is a reflection of its function, i.e., the conduction of excitatory impulses over long distances and the central processing of information from the periphery. The polarity of the nerve cell is manifested in its processes – the dendrites and axon (neurite). At first sight these processes appear to be like the components of any other cells. However, the often extraordinary dimensions of these processes create the problem of integrating them into the overall metabolism of the nerve cell, supplying them with structural constituents and substrates, and regulating molecular events within them. Since the volume of the axon alone may be several hundred times that of the perikaryon and the principal constituents of cell structure, proteins, and lipids have a limited biologic half-life, the continuous replacement of these materials is essential. This is accomplished by the slow axoplasmic transport of materials, whose full significance was first recognized by Paul Weiss. This slow axoplasmic flow, which is most evident in a growing or regenerating nerve fiber, thus serves to maintain the integrity of the axoplasm and is the basis for the maintenance of constructive metabolism in the axon.

An analogous flow is present in the dendrites, which must also be constantly supplied with structural constituents. Dendritic transport appears to be based on a mechanism similar to that of axonal transport (Schubert et al. 1972; Schubert and Kreutzberg 1975b; Gross 1975).

Superimposed on the slow axoplasmic transport of materials is a rapid transport component. This mainly involves membrane constituents, transmitter substances, and enzymes involved in transmitter metabolism. Thus, rapid axonal transport supplies the axolemma and intraaxonal membrane system as well as the synaptic region with structural components and functionally important molecules or their precursors.

Let us return to the principles of cellular membrane dynamics and compartmentalization mentioned earlier. They are valid for all cells and all cells utilize transport processes to achieve them. Ribosomes must be transported to membrane cisternae, secretory granules to the apex of glandular cells, phagosomes to lysosomes, and chromosomes during mitosis. In the nerve cell, such transport mechanisms have reached their culmination, enabling the transport of materials to distant regions of the cell.

This transport and the structures that make it possible enable the nerve cell body to maintain contact with its processes over considerable distances and to respond to a wide variety of changes. The processes of compartmentalization in the nerve cell body must also have attained a higher degree of complexity than in nonneuronal cells. Specifically, a distinction must be made between materials intended to remain in the perikaryon and those destined for axonal and dendritic transport. Furthermore, such transport materials must be coupled to a system which can carry out the transport.

The question of axonal autonomy with respect to the perikaryon is important from both a theoretical and practical clinical standpoint. Despite extensive research material, this question remains unresolved. If the axon is separated from its cell body, the slow axoplasmic flow is halted immediately (Frizell et al. 1975), while rapid transport continues undisturbed for a time (Ochs and Hollingsworth 1971). Eventually, however, the axon succumbs to secondary Wallerian degeneration. Even if it maintains contact with the nerve cell body, the axon can undergo pathologic changes such as distal axonopathy or neuroaxonal dystrophy with no apparent causal connection with the perikaryal metabolism. The situation becomes even more complex if we consider axoglial interactions. In Wallerian degeneration, the myelin-forming cells are not primarily involved; nevertheless, the myelin sheaths are destroyed. In primary lesions of the glial cells or their myelin sheaths, the axons remain undamaged. But demyelinating disease could also arise from a neuronal disorder; this possibility cannot be excluded, since it is known that axons determine whether the glial cells form myelin sheaths (Aguayo et al. 1976b). It would be unlikely in such a case, however, for such a neuronal disorder, which can exist in the absence of an axonal factor, to have morphological manifestations. Disturbances of paranodal contacts between the axolemma and glial cell membrane, called axoglial junctions, could also cause myelination disorders. Waxman and Quick (1978a) have observed a specific affinity for ferrocyanide stain in the node of Ranvier. Neither normal unmyelinated fibers nor demyelinated fibers in dystrophic animals display this affinity (Waxman 1978). This demonstrates that the membrane properties are different in myelinated and un- or demyelinated axon segments.

Besides interactions which are reactive in nature or involve membrane structures, the axon and glia also interact through the direct exchange of materials. These processes are the subject of the glia-neuron-protein transfer hypothesis of Lasek et al. (1974, 1977) and of studies by Elam (1975), Giorgi et al. (1973), and Droz et al. (1978), which suggest that myelin structural components originate from the axon.

Another area of axoglial relationships pertains to regenerative processes in the nervous system. It is well known that the periaxonal cells play a role in the ability of the peripheral nerves to regenerate and in the inability of central nervous pathways in higher vertebrates to functionally regenerate. This role is still poorly understood, however.

These aspects of the biology and pathology of the axon form the framework of the present investigations, which were performed on the optic nerve of the goldfish. This is a central nervous fiber system which has already served as a model in numerous experimental studies of axonal transport (e.g., Grafstein and Murray 1969), regeneration (e.g., Murray 1976), and retinotectal connections (e.g., Attardi and Sperry 1963). The goals of our investigation were (1) to study axonal transport under normal and cytopathologic conditions; (2) to investigate axoglial interactions; (3) to trace morphological changes following the interruption of fiber continuity at various levels in the optic system; and (4) to observe the regeneration of this fiber system. The latter was

done by two different experimental methods. The first method is well known and is the one most commonly employed in regeneration studies: crushing of the optic nerve and the observation of subsequent axon growth through the crush zone into the optic tectum. The second method is based on the recently discovered ability of the fish retina to regenerate following damage or removal. The axons of the newly formed ganglion cells of the optic nerve ganglionic layer grow into the old optic nerve and form new functional contacts in the optic tectum. Since both experiments can be performed on the two optic nerves of the same animal and the growth of the axons of the new retinal ganglion cells is more or less synchronous with contralateral axon regeneration, it is possible to make direct comparisons both with regard to the degeneration of the optic nerve and the associated glial activation and connective tissue reaction, as well as the remyelination of the regenerating axons and synaptogenesis in the optic tectum (cf. Wolburg 1975, 1976a, 1978).

2 Material and Methods

Experimental Animals. Goldfish (*Carassius auratus*) and crucian carp (*Carassius carassius*) were obtained from a local zoological supply house. Most of the animals had a body length of 12–14 cm; some were smaller (6–9 cm). The animals were stored in a 200-l aquarium, fed with Tetramin, and transferred to smaller tanks during the period of the experiment.

Injected Substances. Drugs and radiolabeled RNA precursors and protein precursors were injected intraocularly.

Ouabain: Serva, 10 µl, 10^{-4} *M*, 10^{-5} *M*, 10^{-6} *M*, dissolved in 0.65% NaCl. The final intraocular concentration was between 2 and $3 \cdot 10^{-5}$ *M* (10^{-6} *M*, 10^{-7} *M*, respectively.

^{3}H-uridine: Amersham-Buchler, 10 µCi, sp. act. 8–12 Ci/m*M*.

^{3}H-proline: Amersham-Buchler, 10 µCi, sp. act. 17–22 Ci/m*M*.

In many experiments 0.65% NaCl solution was injected contralaterally as a control.

Injections. The substances were injected intraocularly with a Glenco microliter syringe. The eyeball was immobilized with a perforated template, and the cannula was inserted through the perforation laterally into the posterior chamber. The injection began when the cannula opening could be seen through the pupil and lens against the dark eyeground. After the injection was completed, the cannula was left in place for a few seconds to permit distribution of the injected fluid and to equalize pressures.

Specimens for Liquid Scintillation Measurements. For radioactivity measurements, the tissue was treated with 10% trichloracetic acid (TCA). Following decapitation, the brain was taken from the skull, freed of fat and connective tissue, and all brain parts removed except for the visual system and cerebellum. The visual system was subdivided into four segments: the optic nerve from the eye base to the chiasm formed two segments; the optic tract between the chiasm and tectum formed the third segment, and the anterior half of the tectum the fourth segment. In several in

vitro experiments using animals 6–9 cm long, the optic nerve was dissected out in its entirety so that a total of only three segments were obtained. The cerebellum served as a control tissue unassociated with the retina and thus provided a means of monitoring the label in the blood stream. The tissue samples were weighed and either treated with 10% TCA at 4 °C for 24 h to determine the TCA-resistant compounds, or immediately processed for measurement of total radioactivity. The samples were dissolved overnight in scintillation vials containing 0.5 ml Protosol (NEN Chemicals); the specimen was then treated with 10 ml toluene scintillator [5 g PPO (2,5-diphenyloxazole) and 0.5 g POPOP (1,4-bis-2-(5-phenyloxazolyl) benzene) to 1 l toluene] or Instagel (Packard) and measured in a liquid scintillation counter (Packard 3320 or Beta-Scint BF 5000). The measured values (counts per minute, cpm), from which the background was subtracted, were related to milligram fresh weight.

Method for In Vitro Experiments. The visual system was dissected as free as possible from other brain parts and connective tissue and transferred to the incubation vessel of the in vitro apparatus. The medium consisted of Eagle's Minimum Essential Medium with Earle's salts (Serva). Carbogen gas was bubbled through the medium in the stock container, and the overpressure was vented directly into the incubation vessel. This was done to offset any oxygen loss from the carbogen-presaturated medium on the way from the stock bottle to the incubation vessel. Also, the gas stream created a turbulence in the incubation vessel which kept the tissue in constant motion and improved the diffusion conditions for oxygen and nutrients.

Retrobulbar Crushing of Optic Nerve. Fiber regeneration in the goldfish visual system was investigated following two different procedures in the animal (Fig. 1). First, ouabain was injected intraocularly to produce retinal destruction and subsequent regeneration. The axons of the regenerated retinal ganglion cells grew through the old optic nerve into the tectum. This process of cellular regeneration and axon growth was compared with the axonal regeneration of proximal fiber stumps secondary to crushing of the contralateral nerve in the same animal. The nerve crush was carried out in the anesthetized animal: 0.02% MS 222 (Sandoz) was added to the water, and in 3–4 min the animal could be removed and treated. The connective tissue around the eyeball was sectioned to permit ocular rotation. After the removal of fatty and connective tissue, the optic nerve insertion became directly visible as a white spot and accessible. The nerve was grasped as close to the eyeball as possible with a fine watchmaker's forceps and forcibly squeezed for about 5 s, care being taken to avoid complete transection. In most cases there was no bleeding; the crush zone itself was about 1 mm wide. The duration of the experiments was 2, 4, 8, 14, 16, 19, 28, 29, 35, 38, 43, 47, 51, 57, 60, 73, and 85 days (one animal in each case, with the following exceptions: 2, 4, 14, and 57 days for two animals and 8 days for three animals). After the experiments were completed, the nerve was separated from the eye directly behind the crush zone, if still visible. In addition, the trauma zone itself was studied in longitudinal sections in four animals 5, 6, 8, and 14 days after bilateral crushing.

Light and Electron Microscopy. The retina, nerve, and tectum were brought in contact with fixative as soon as possible during dissection. Fine dissection and the trimming of tissue pieces under the stereoscopic microscope were also carried out in fixing solution. The primary fixative was 2% glutaraldehyde (Polysciences) buffered with

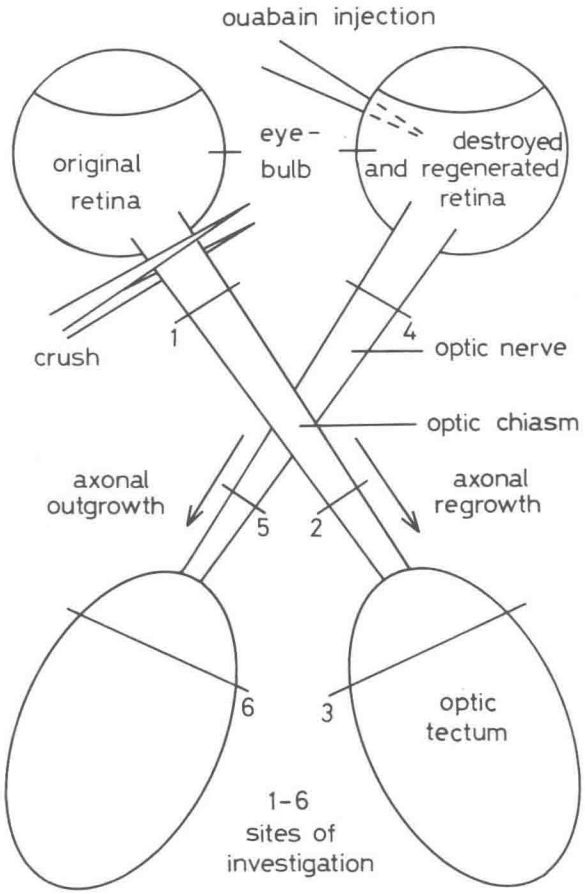


Fig. 1. Schematic representation of operative procedures for degeneration and regeneration experiments in the visual system of the goldfish. See Materials and Methods for further details

0.1 *M* cacodylate buffer. The fixation time was 40–50 min. The tissues were washed in cacodylate buffer with 0.2 *M* sucrose added (washing buffer), followed by 1-h postfixing in cacodylate-buffered 1% O_sO_4 solution, washing for 30 min in washing buffer, and dehydration with 50% and 70% alcohol. The tissue then was treated with uranyl acetate-saturated 70% alcohol overnight in the refrigerator and then dehydrated further with 80%, 96%, and absolute alcohol and propylene oxide. Using Araldite-propylene oxide mixtures in a ratio of 1:1 and 3:1, the tissue was finally embedded in Araldite (Ciba) using a flat silicone embedding mold. The specimen was polymerized for 2 h at 50 °C or 60 °C and 24 h 90 °C, then cut with an OMU 3 ultramicrotome (Reichert) into semithin sections, 1 μm thick, which where stained with toluidine blue and into ultrathin sections which were stained with lead citrate. The sections were examined with an Elmiskop 102 (Siemens) and an EM10 (Zeiss) electron microscope and photographed with Scientia film (Agfa-Gevaert).

3 Results

3.1 Morphology of the Goldfish Visual System

3.1.1 Retina

The ganglion cells of the optic nerve ganglionic layer are the perikarya of the optic axons (Fig. 2b, c), which compose the optic nerve and pass to the optic tectum. In the retina (Fig. 2a) they are connected with the bipolar cells via complex synapses in the inner plexiform layer, and the bipolar cells with the receptor cells of the outer nuclear layer, the rods and cones. These nervous elements are connected horizontally by amacrine and horizontal cells, which together with the bipolars are contained within the inner nuclear layer. In the goldfish the retina continues to grow throughout life. Johns (1977), Johns and Easter (1977), and Meyer (1978) have determined the growth rates for each cell type in the goldfish retina. Retinal cell growth proceeds from a marginal zone near the ora serrata, the pars ciliaris retinae (anterior complex, Keefe 1973; Fig. 2a). This epithelium is very active mitotically and continuously produces new retinal cells. Mitoses and ^{3}H-thymidine incorporation are also observed in the outer nuclear layer of the retina (Maier 1978). The outer nuclear layer and the pars ciliaris retinae are the two sources of retinal tissue regeneration following damage by ouabain (Maier 1978; Maier and Wolburg 1978, 1979). The optic axons within the retina are sparsely myelinated or unmyelinated (Fig. 2c; Wolburg 1980). The oligodendrocytes are sparsely but uniformly distributed throughout the retina, embedded among the bundles of ganglion cell axons. The latter are directly adjacent to the inner limiting membrane, which is bounded on the vitreous side by blood vessels (Fig. 2c). The retina itself is avascular. No fovea centralis could be detected.

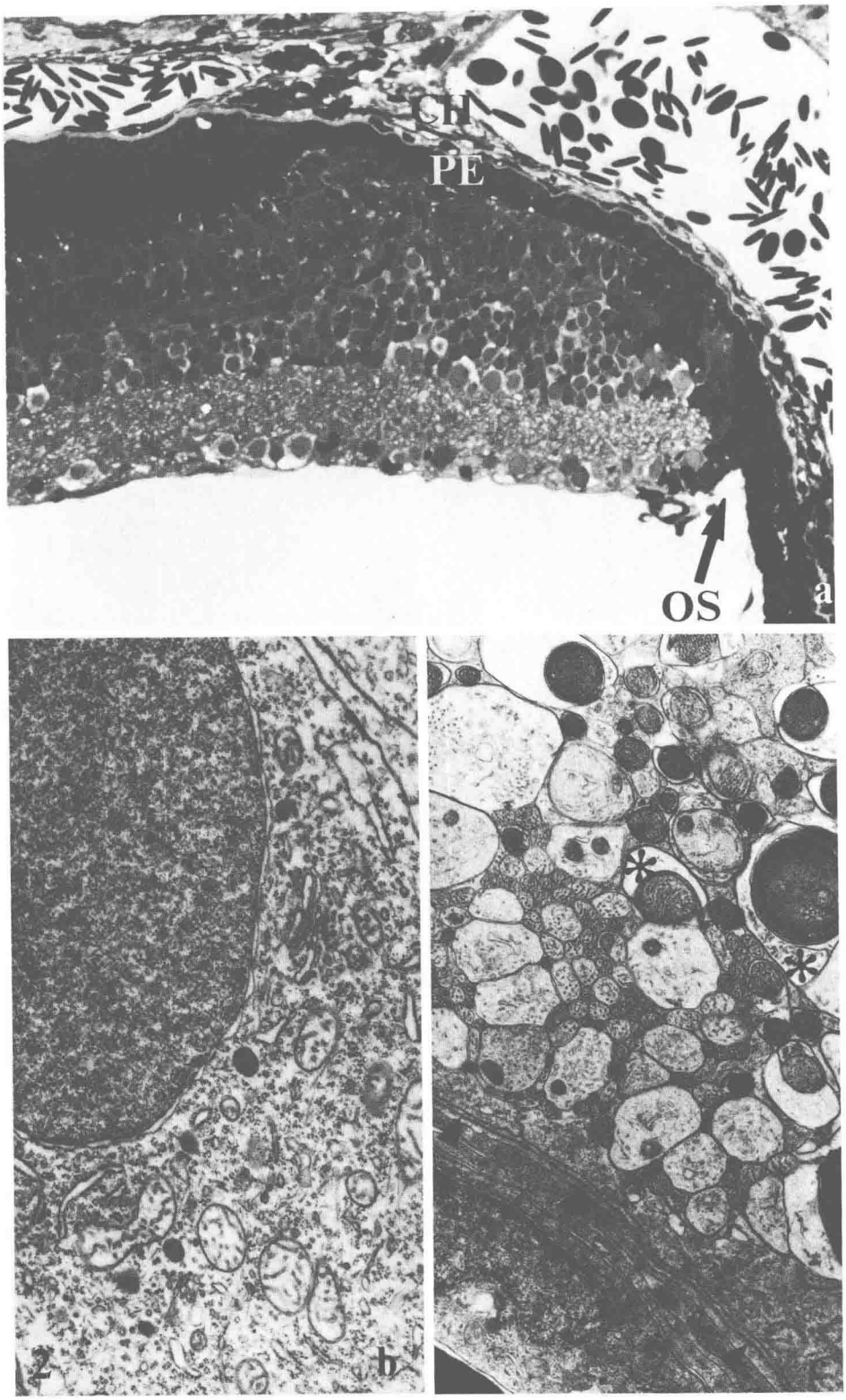
3.1.2 Optic Nerve and Optic Tract

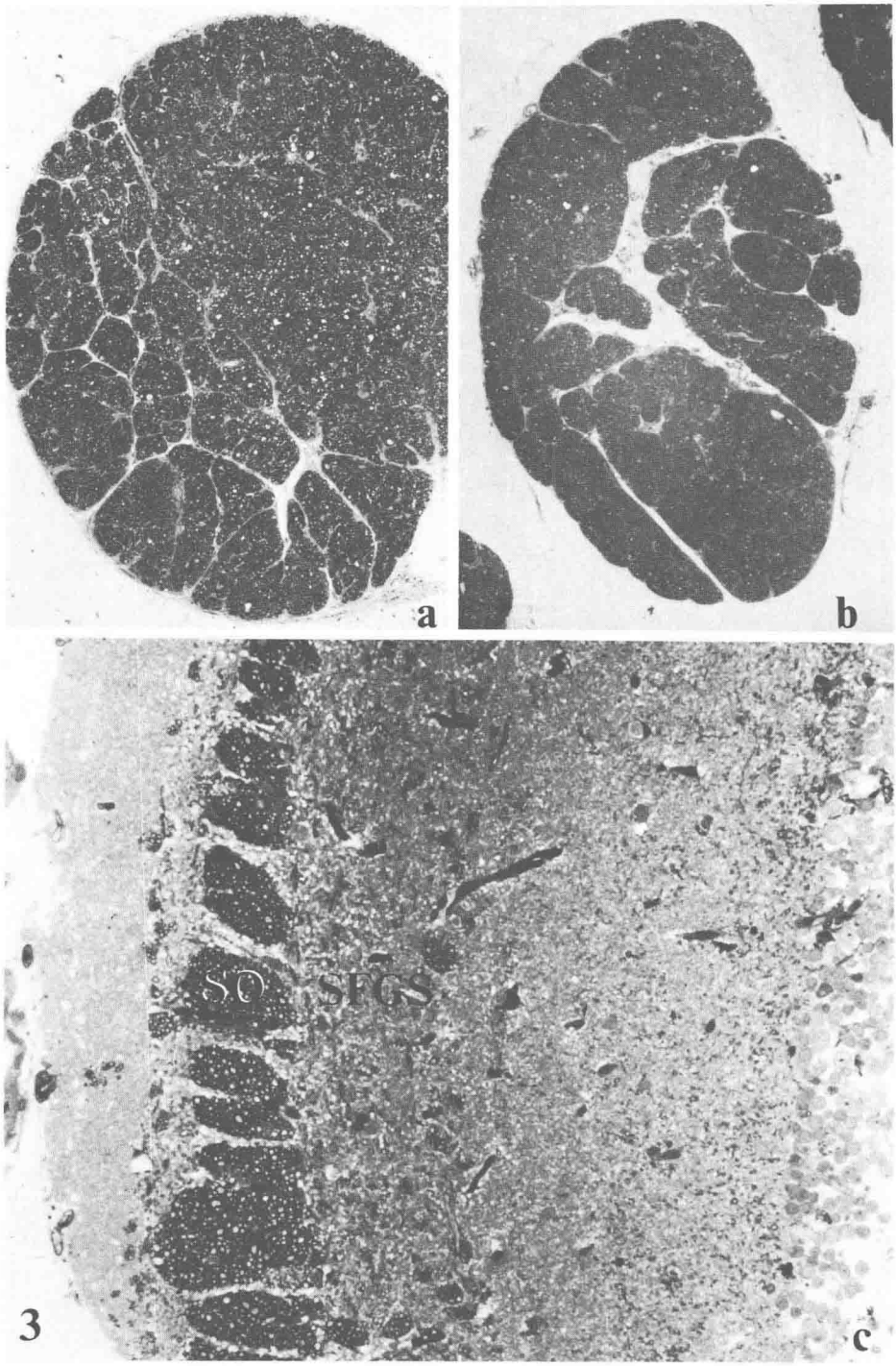
At the point where the nerve emerges from the eye, the firm sclera merges with an equally firm primitive meninx, which is not layered in fish. It extends to the optic chiasm. Between the optic chiasm and optic tectum the optic tract is ensheathed only by a very delicate layer of connective tissue. In the optic chiasm the two optic nerves are completely crossed. The question of which nerve is overriding is an individual variant.

The great majority of axons in the optic nerve are myelinated (Fig. 3a). The caliber of the fibers ranges from about 0.5 μm to 2 μm, though individual axons may be larger; the distribution pattern is unimodal. The fiber calibers can vary considerably in the different fascicles of the anterior nerve segment. Thus, fascicles containing predominantly axons 1-μm thick may alternate with fascicles in which the axon calibers are mainly in the 2-μm range. While the caliber may be quite uniform in one fascicle,

---

Fig. 2a–c. Normal goldfish retina. *a* Marginal growth zone (pars ciliaris retinae, anterior complex) at ora serrate (*OS*). *PE*, pigment epithelium; *CH, chorioidea,* × 440, *b* Nerve cell of ganglionic layer of optic nerve, × 15 000, *c* Retinal fiber layer bordering vitreous, showing axons and outer loop of oligodendroglial cytoplasm (*); *arrowheads,* internal limiting membrane, x 4500


CH
PE
OS
a
2
b


a
b
SO
SFGS
3
c

another may contain very small fibers (0.5 μm) alongside very large ones (4 μm or more). The fascicles are separated from one another by connective tissue septa containing fibrocytes, collagenous fibers, and blood vessels, while the fascicles themselves are subdivided by astrocytes and separated from the connective tissue by an astrocytic basal lamina. These boundary zones contain numerous desmosomes which interconnect adjacent astrocytes. The astrocytes are recognizable by their filaments and large, light nuclei. The myelinated fibers are densely packed; the outer loop of oligodendroglial cytoplasm is often visible only as a narrow seam and is sometimes so thin that the fibers appear to be ensheathed only by astrocytes. The oligodendrocytes have a darker and denser cytoplasm and nucleus than the astrocytes; they possess microtubules, few microfilaments, and are rich in organelles (rough endoplasmic reticulum, Golgi complex). They are fewer in number than the astrocytes. The axons contain microtubules, neurofilaments, mitochondria, and membrane profiles of smooth endoplasmic reticulum.

In the optic tract the subdivision into fascicles is less distinct than in the optic nerve (Fig. 3b). The optic tract displays a more uniform cross section, and glial cells are less numerous than in the optic nerve.

3.1.3 Optic Tectum

The optic tectum is the most prominent part of the fish brain and is the site where all afferent fibers from the retina are processed or relayed. The tectum consists of a pair of semiellipsoid structures which (in the 1-year-old goldfish with a body length of about 7–8 cm) measure 5 mm rostrocaudally and about 3 mm lateromedially. The surface is smooth and invested by a highly vascular meninx. The tectum can be subdivided into various layers on cross section (Fig. 3c): Near the surface is the stratum marginale, which is almost free of cell bodies and is filled with neuropil. Below it is the stratum opticum, which contains predominantly, but not exclusively, the axons of the retinal ganglion cells. It can be subdivided into a marginal part with small fiber groups (pars superficialis) and a deeper, larger part called the pars profunda. Below this is the stratum fibrosum et griseum superficiale, which contains mainly the unmyelinated terminal portions of the optic axons and their synapses with central neurons. Nerve cell bodies are also found in this layer. Next follows the extensive stratum griseum centrale, then the stratum album centrale, which contains numerous myelinated axons, and finally the stratum periventriculare, which is rich in cell bodies. Dendritic trees rise from these cells to the tectal surface. From the stratum album centrale, bundles of fibers course through the periventricular zone and across the ventricle into deeper regions of the tegmentum. The optic fibers of the stratum opticum are not uniformly distributed over the cross section of the tectum. The optic tract divides into two main parts upon entering the tectum: The first passes ventrolaterally along

---

Fig. 3a–c. Normal fiber system of the goldfish. *a* Cross section through optic nerve about 3 mm distal to eyeball. Connective tissue septa irregularly permeate the nerve; × 100, *b* Cross section through the optic tract about 7 mm distal to eyeball; × 100, *c* Cross section through the optic tectum. Only the pars profunda of the stratum opticum (*SO*) contains retinotectal projections; the stratum fibrosum et griseum superficiale (*SFGS*) contains the axodentritic synapses, the sites of retinotectal connection; × 280