Functional Regeneration of the Visual System in Teleosts. Comparative Investigations after Optic Nerve Crush and Damage of the Retina

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Goldfish and carp are shown to be capable of functional reconstitution of the visual system, although their neural retina except of the marginal growth zone at the ora serrata was completely destroyed by injection of the Na-K-ATPase inhibitor ouabain. This was demonstrated by the observation of the recovery of the optokinetic nystagmus after damage of the retina. Recovery of vision coincides well with the morphological reconstitution of the visual system. The regenerated axons within the optic nerve are still unmyelinated at the moment of visual recovery, whereas some fibers within the stratum opticum pars profunda of the corresponding optic tectum are already myelinated. The recovery of vision after regeneration of the retina was compared with the recovery of vision after crush of the optic nerve. The range of time needed for visual recovery was smaller and better reproducible after crush than after damage of the retina.

Introduction

There is a lot of investigations dealing with the capability of the fish visual system to regenerate after damage. Many of these studies involve mapping techniques [1–6] or behavioral tests [4, 7, 8], others are confined to morphological observations [9–12]. In the present study we report a method to prove the recovery of a visual capability and the visual acuity after damage of the visual system. This method is based on the optokinetic nystagmus [7, 13], which can be observed directly in the optokinetic drum. Furthermore, we establish for the first time the functional regeneration of the retina of goldfish and crucian carp after ouabain-induced destruction. The morphological regeneration of the retina in fish is well established [12, 14, 15]; the functional aspect of this phenomenon was not investigated as yet.

The results were presented in a preliminary form elsewhere [16].

Materials and Methods

50 goldfish (*Carassius auratus*) and 25 crucian carp (*Carassius carassius;* body length 8 cm) were used in the present study; the animals were divided into seven experimental groups: (1) crushing the optic nerve once; (2) crushing the optic nerve twice; both lesions (first: conditioning lesion, second: test lesion) had a time difference of fourteen days; (3–6) intraocular injection of 5 µl (3) 10^{-6} M, (4) 5×10^{-6} M, (5) 10^{-5} M, (6) 10^{-4} M ouabain; (7) crushing the optic nerve after first recovery of vision following an injection of 5 µl 10^{-5} M ouabain. All animals were tested for visual capability using the optokinetic nystagmus.

The *optokinetic nystagmus* was observed directly by eye without special recording. An optokinetic drum (diameter 33 cm) coated with black and white stripes rotated around a circular aquarium (diameter 12 cm) in which individual fish were restrained. Six different sizes of stripes were used: 30, 10, 5, 2, 1, 0.5 mm, corresponding to a visual angle of 17°, 6°, 3°, 1°, 35′, 17′. The rotation velocity was 5 rpm, corresponding an angular velocity of 30°/sec. The daily test of the respective animals began 6 to 8 days before the recovery of vision could theoretically be expected.

Operative procedures: All operative procedures were made bilaterally during anesthetizing in 0.02% MS 222 (Sandoz). An incision of the connective tissue around the eye was carefully made in order to reach the optic nerve more easily with watchmaker forceps. Then the optic nerve was crushed for about 4 sec directly behind the eye bulb. Special care was taken to avoid bleeding and damage of the eye-muscles. The intraocular injection of the ouabain-

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solution (Ouabain, Serva, in 0.65% NaCl) was carried out with a Glenco microliter syringe.

Electron microscopy: At different points of the regeneration process (before, at and after the moment of recovery of vision) several animals of the experimental groups 1, 5 and 6 were taken from the running experiment to assay the morphology of the visual system. The animals were decapitated and the retina, optic nerve, tract and tectum dissected out, fixed by immersion in 2% glutaraldehyde (Polysciences) buffered in 0.1 M sodium cacodylate buffer (pH 7.2) without surcrose, washed in the same buffer with 0.2 M sucrose, postfixed in 1% buffered OsO_4, rinsed in washing buffer again and dehydrated by alcohol and propyleneoxide. For the contrast enhancement the block was emerged in 70% alcohol saturated with uranylacetate overnight and finally embedded in Araldite (Ciba). Semithin sections were stained with toluidine blue, ultrathin sections with lead citrate.

Results

Optokinetic nystagmus

Control animals: The nystagmus could be observed to an angle of 17′. However, there was individual variability of response (3° to 17′). Nevertheless, the results coincide largely with other investigations about visual acuity in fish [13].

After *retrobulbar crush* of the optic nerve the optokinetic nystagmus could not be observed for a time of 23 to 45 days (Fig. 1 a). From the 20th day after crush the animals were tested every day. At the moment of recovery of vision the visual acuity was less than some days later (6° − 1°). Finally, the visual acuity reach normal values (3° − 17′; Fig. 2). Conditioning experiments have confirmed the findings of McQuarrie and Grafstein [17] and Edwards *et al.* [8] who found that the velocity of axonal regrowth after crushing the optic nerve can be increased by a first (conditioning) lesion 14 days before the second (test) lesion. In fact, with the method of optokinetic nystagmus we found shortening of the time between test lesion and recovery of vision of about 9 days when using the conditioning lesion (Fig. 1 b).

Injection of 5 µl 10^{-6} M *ouabain* does not cause any blindness, but a decreased visual acuity (3° − 6°) for 10 − 15 days.

After *injection of* 5 µl 5×10^{-6} M *ouabain* the animals were blind for 1 − 2 days (Fig. 1 c). Apparently, this type of blindness was not based on the destruction of retinal cells, but can more easily be explained as a reversible effect on the ganglion cells which might be uncapable for short time to produce action potentials.

After *injection of* 5 µl 10^{-5} M *ouabain* the time of blindness ranged from 32 to 103 days. The high variability did not permit statistical analysis, because the number of animals tested was too small (Fig. 1 d). Nevertheless, the average time of 53 days coincides well with the morphological observations about reappearance of synapses in the tectum described by Wolburg [12]. The visual acuity at the moment of vision recovery was clearly less than normal (Fig. 2), but the measurements were difficult because of dimming of the lens in some cases caused by the injection procedure.

In addition, we wanted to know if the axon growth from *new* ganglion cells may be faster then the growth of regenerating axons from preexisting ganglion cells. Therefore, we crushed the optic nerve behind the eye-bulb when the "fastest" animal of the appropriate experimental group just had recovered from retinal damage and blindness. The time necessary for a second recovery of vision was less than after crush only; however, the effect was not as clear (Fig. 1 e) as seen after two crushes (Fig. 1 b).

After *injection of* 5 µl 10^{-4} M *ouabain* the time of vision recovery was very long because of a total destruction of the differentiated neural retina (87 to 106 days; Fig. 1 f). Thus, the long delay of vision recovery can be correlated to the relatively long period of retina regeneration, which preceeds axonal growth and synaptogenesis. Also in this experiment the visual capability after recovery of vision was difficult to determine because of the dimming of the lens.

Morphological findings

Systematic investigations about the morphological alterations in the optic system during both Wallerian degeneration and the regeneration were published by Wolburg [12]. In the present study only few morphological observations at selected times of the regenerative process were made in order to correlate structural aspects with the functional stage of the visual system.

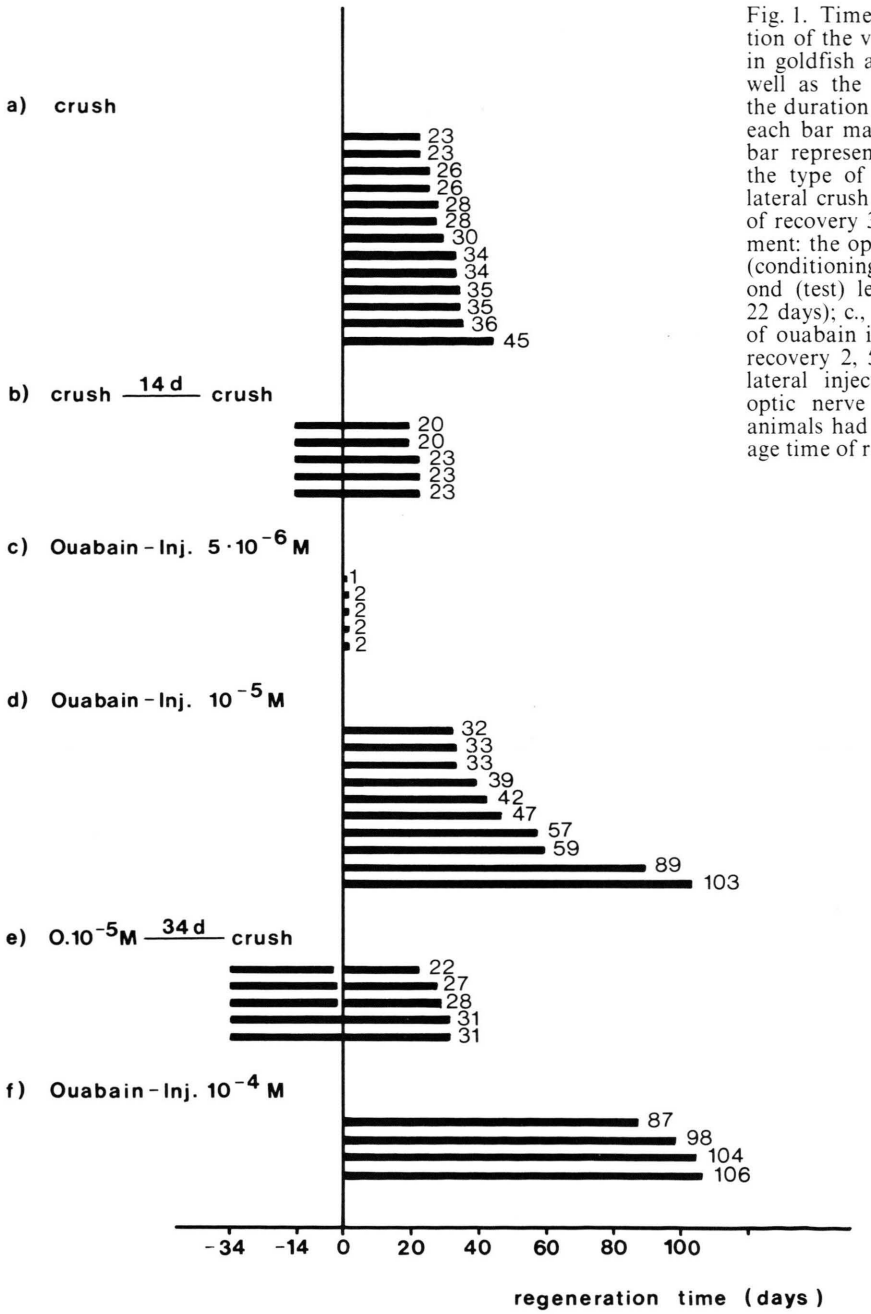


Fig. 1. Time needed for functional regeneration of the visual system (recovery of vision) in goldfish and carp. The horizontal bars as well as the numbers behind them indicate the duration of blindness in days. The end of each bar marks the recovery of vision. Each bar represents one animal. On the left side the type of experiment is indicated: a. bilateral crush of the optic nerve (average time of recovery 31 days); b. conditioning experiment: the optic nerve was crushed bilaterally (conditioning lesion) 14 days before the second (test) lesion (average time of recovery 22 days); c., d., f. injection of different doses of ouabain into both eyes (average times of recovery 2, 53, 99 days, respectively); e. bilateral injection of ouabain, crush of the optic nerve 34 days later when the first animals had recovered from blindness (average time of recovery 28 days).

Crushing of the optic nerve is followed by Wallerian degeneration; retrograde degeneration of the retina was never observed. Wallerian degeneration is associated with proliferation of both astroglial and oligodendroglial cells. Several days before the recovery of vision was expected the optic nerve and tract contains thin, completely unmyelinated nerve fibers, bundled and embraced by glial processes. In the optic layer of the tectum which contains the regenerated optic fibers, only a very small portion of the fibers are myelinated. At the moment of recovery of vision the optic nerve and tract are

unaltered (Fig. 3), the portion of myelinated regenerated optic fibers in the tectum is slightly increased (Fig. 4). Thus optic axons, which are destined to be myelinated are capable of impulse conductance *before* myelination.

The regeneration of the retina after ouabain injection has been described in detail by Maier and Wolburg [14]. Dependent on the degree of damage, whether the outer nuclear layer was completely destroyed or not, the time for regeneration and, correspondingly, the time for recovery of vision, were different. Fig. 5 shows the retina 41 days after injection of 5 µl 10^{-5} M ouabain and 2 days after recovery of vision.

The morphological findings after ouabain injection refering to regeneration, remyelination and the disto-proximal gradient of myelination from the optic tectum to the nerve in relation to the recovery of vision correspond to those after crush. Only the moment of recovery of vision is different because of the larger time lag of retina regeneration.

Discussion

This is the first report on the functional regeneration of the retina in teleosts. The reappearance of optokinetic nystagmus was chosen as criterion of functional regeneration. Other, less precise tests such as recovery of food localization, of the startle reaction or the lightening of the melanophores in the skin, could be correlated well with the nystagmus test. What could be only assumed by morphology up to now [12] is now proven: (1) although complete destruction of the neural retina – except for its margin – the animals restitute their visual capability, (2) the range of time for the recovery of vision is smaller and better reproducible after crush than after ouabain injection. Technical reasons are responsible: the inevitable reflow of the injected substance and the variable distribution of the drug in the eye-bulb are reflected not only by a different degree of damage but also by a different delay of recovery of vision.

The conditioning effect of a first lesion of the optic nerve 14 days before a second test lesion upon the time of recovery of vision, as documented by McQuarrie and Grafstein [17] and Edwards *et al.* [8], could be confirmed in this study. This effect is presumably based on two different mechanisms, (1) the reduction of the initial delay before the outgrowth of axons starts [17] and (2) the increase of the velocity of axonal growth [17, 18]. Moreover, we have envisaged the possibility that a newly established ganglion cell may be conditioned to regenerate its axon faster than a preexisting neuron. Here, the lesion interval was not 14 days, but the time necessary for the recovery of vision in the "fastest" animal of the experimental group (34 days; Fig. 1 e). The time for visual recovery was slightly reduced (average 28 days) in comparison with the controls (single crush in animals not operated before; average 31 days; Fig. 1 a), but for definitive statistical affirmation the number of animals tested appears to be too small. In any case, the effect was less pronounced than the effect of the conditioning crush (Fig. 1 b). It would be interesting to know if one or both of the two mechanisms mentioned above are responsible for the conditioning effect. Nevertheless the question: "Do young axons regenerate better than old axons?" asked by Goldberg and Frank [19] and denied by the authors for chicken and mice, must possibly be affirmed for the teleost optic axons.

The moment of recovery of vision may be nearly identical with the moment of synaptogenesis of most optic terminals in the tectum. It is an important finding that at the moment of recovery of vision *all* fibers in the optic nerve are totally unmyelinated. This is in accordance with recent results established in the developing optic system of the rat by Foster *et al.* [20]. The fact of impulse conduction by unmyelinated fibers which subsequently will become myelinated indicates that after the first period of continuous impulse conduction a fundamental reorganisation of the axonal membrane may occur (compare Wiley-Livingston and Ellisman, [21]).

Concerning the direction of myelination it was reported by Wolburg [12, 22] that at least under conditions of regeneration the first myelin sheaths develop in the tectum and later on in the optic nerve (Figs. 3, 4). This would be indicative for retrograde direction of myelination. These results are reconfirmed in the present study. Theoretically, non-optic fibers or collaterals from other than retinal sources in the stratum opticum pars profunda of the goldfish optic tectum could interfere with these observations. The unequivocal proof of retrograde myelination can be expected only by tracing labelled regenerated yet unmyelinated optic fibers from the optic nerve to the myelinated fibers seen in the tectum.

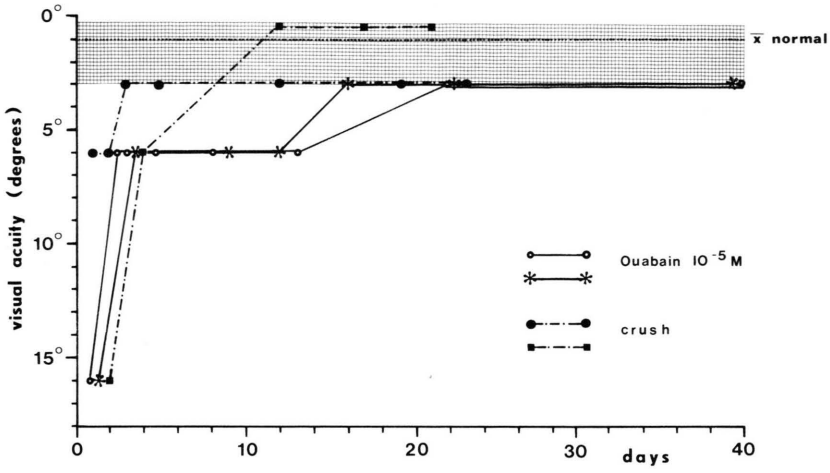


Fig. 2. Representative examples of the development of visual acuity after recovery of vision in two animals after ouabain injection and crush, respectively. Time zero means the moment of recovery of vision. The visual acuity becomes normal in most of the experimental animals, after crush faster than after retina regeneration. The range of visual acuity found in control animals is hatched. The average visual acuity is 1°.

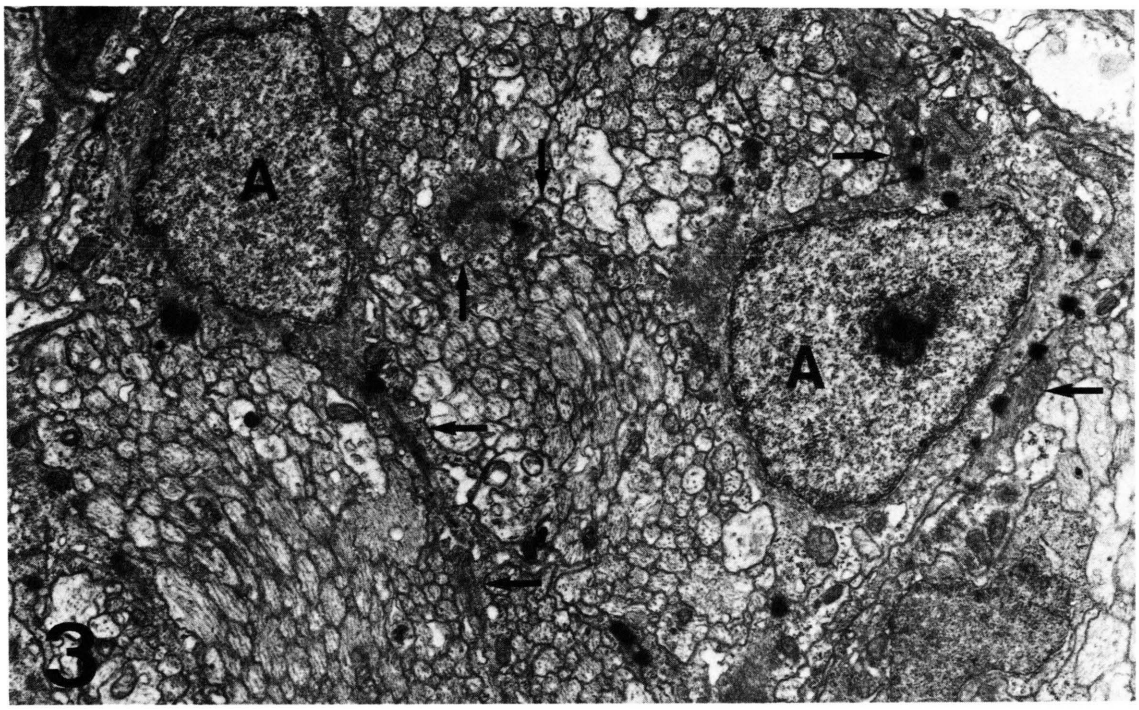
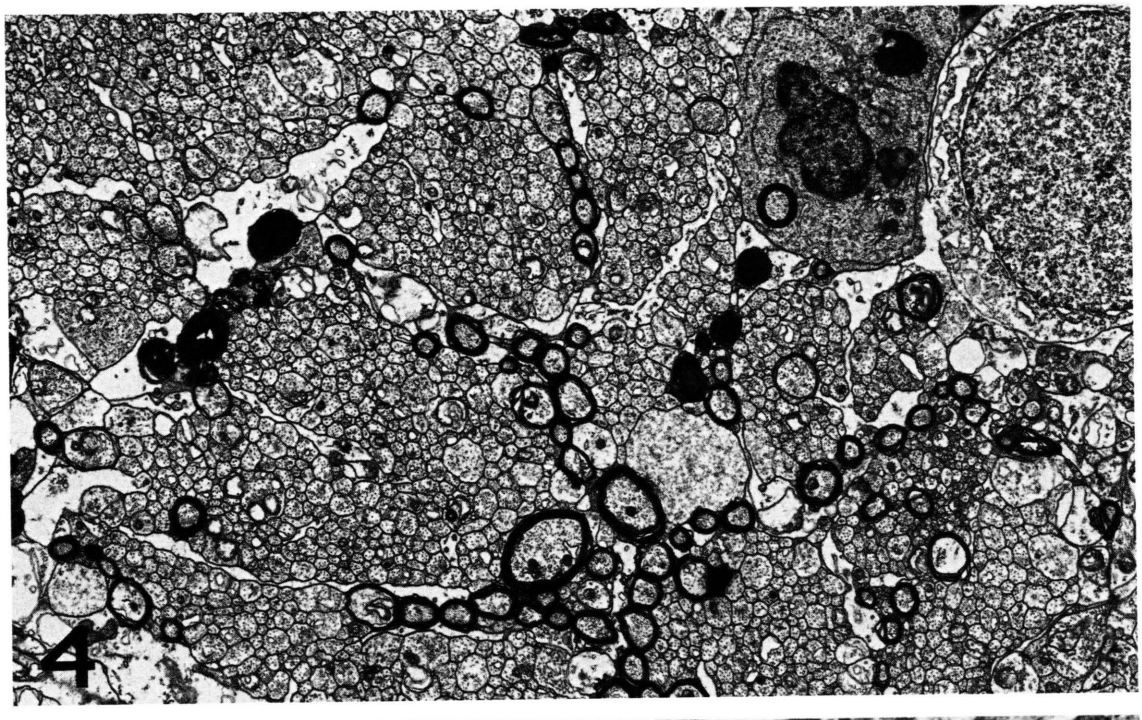


Fig. 3. Transversal section of an optic nerve, 55 days after crush and 22 days after recovery of vision. The visual acuity of this animal tested with the optokinetic nystagmus was in the normal range (compare Fig. 2); all fibers are still unmyelinated, in contrast to the corresponding optic tectum (Fig. 4). A astrocyte, embracing bundles of regenerated axons by cytoplasmic processes (arrows). 9000:1.

Fig. 4. Transversal section of the optic layer in the tectum whose corresponding optic nerve is shown in Fig. 3. Some of the regenerated fibers are already myelinated, whereas in the corresponding optic nerve no fibers are myelinated (compare Fig. 3). O, oligodendrocyte. 7500:1.

Fig. 5. Light micrograph of a regenerated retina of goldfish, 41 days after intraocular injection of 5 µl 10^{-5} M ouabain and 2 days after recovery of vision. The retina is still thinner than normal, the inner nuclear layer is poorly developed. The corresponding regenerated optic nerve is unmyelinated and looks similar to Fig. 3 after crush. The visual acuity of this animal directly before sacrifice was 16°. OSL outer segment layer, ONL outer nuclear layer, INL inner nuclear layer, arrows indicate the membrana limitans externa. 880:1.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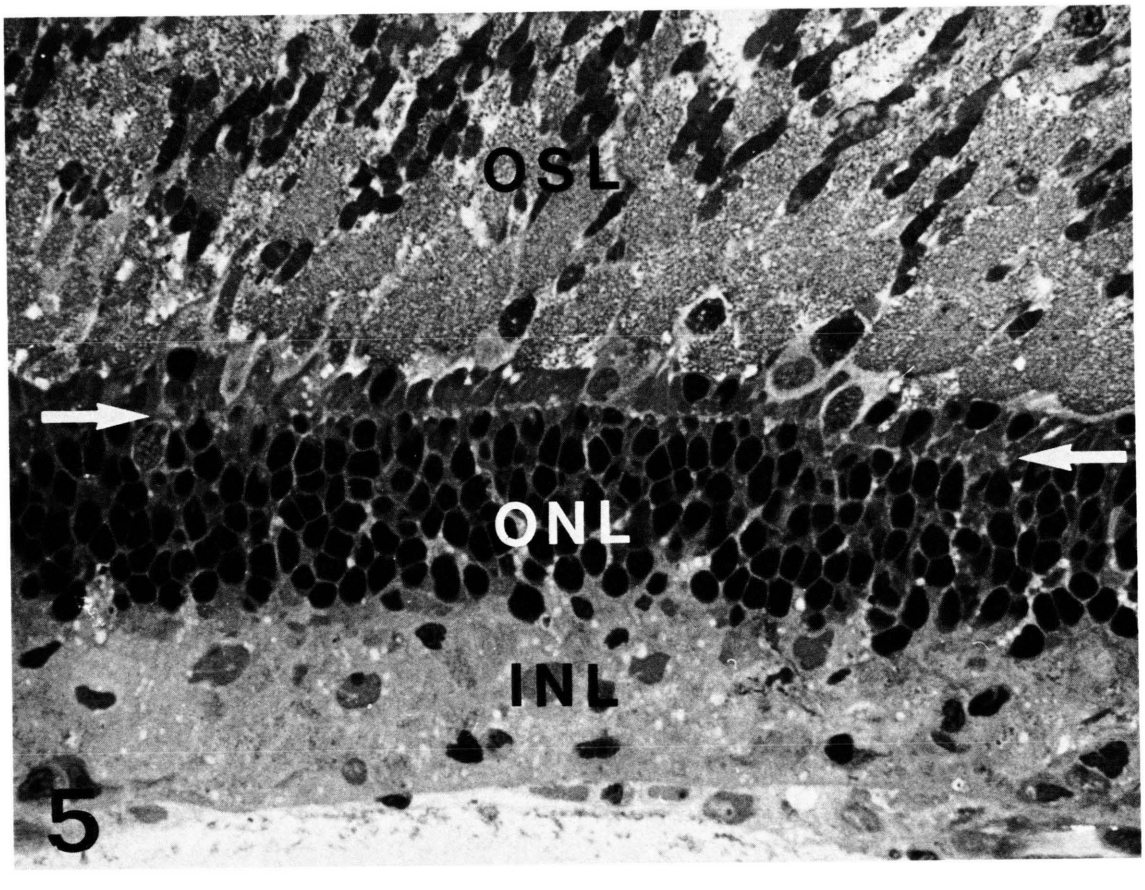
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