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EXPLANT CULTURE OF ADULT GOLDFISH RETINA: A MODEL FOR THE STUDY OF CNS REGENERATION

G. E. LANDRETH and B. W. AGRANOFF

Neuroscience Laboratory, University of Michigan, Ann Arbor, Mich. 48109 (U.S.A.)

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SUMMARY

Conditions are described for culture of retinal explants of adult goldfish which favour outgrowth of neuritic processes onto a substratum. A growth index to quantitate the outgrowth was developed. If the optic nerve is crushed several days prior to explantation, a marked enhancement of neuritic outgrowth is seen relative to control retinas. Histological examination of the explants revealed that retinal ganglion cells in explants from unoperated eyes became hypertrophied in vitro with a time course similar to that observed in vivo following optic nerve crush. Experiments with hemiaxotomized retinas indicate that the perikaryal regenerative response is mediated intracellularly.

INTRODUCTION

The goldfish visual system has been used extensively in studies of central nervous system (CNS) regeneration and plasticity[14,23]. Like many other poikilotherms, the goldfish can regenerate its optic nerve following section, and the amputated retinal ganglion cell axons have been shown to reestablish functional connections with the optic tectum[2,24,48] and the diencephalon[40,49]. In order to examine more directly the events which accompany optic nerve regeneration, methods were developed to maintain the adult goldfish retina in vitro. Agranoff et al. reported that neuritic outgrowth from larval Xenopus retina was facilitated if the optic nerve had been cut in situ several days prior to explantation of the retina[1], and more recently evidence has been reported for enhanced neuritic outgrowth as a result of prior optic nerve section in goldfish retinal cultures[30,31]. In the latter cultures it is now evident that the retinal ganglion cells are the source of the neurites[26,27]. The present study summarizes the optimization of growth conditions for goldfish retinal cultures and describes a convenient growth index. Studies are presented on the nature of the ganglion cell response that leads to the neuritic outgrowth.

MATERIALS AND METHODS

Common goldfish (*Carassius auratus*) 6–7 cm in body length, weighing 7–11 g, were obtained from Ozark Fisheries (Stoutland, Mo.). Fish were maintained in constant light at 21±1 °C.

Surgical procedures

Goldfish were anesthetized in Finquel (MS-222, Ayerst). To crush the optic nerve, the extraocular muscles were cut with a pair of fine scissors, and the eye was pulled forward in the orbit exposing the optic nerve, which was then crushed with forceps leaving the surrounding dural sheath intact. Care was taken not to damage the associated artery. Fish that bled at the site of the crush were discarded. In experiments involving tectal ablation, the cranium was opened along the sutures with a sharp scalpel and the bone flap reflected. The right caudal half-tectum was aspirated following division of the tectum with a pair of fine scissors. The bone flap was replaced and tissue adhesive (Ethicon) was applied at its edges.

Histology

Tissue was prepared for histological examination by fixation for 48 h in 80% ethanol–formaldehyde–acetic acid (18:1:1) at room temperature, then embedded in paraffin and cut in 8 μm sections before staining with cresyl violet or hematoxylin–eosin.

Media and substrata

Phosphate-buffered saline (PBS) was prepared according to Dulbecco[11]. The nutrient medium was that of Leibovitz[33] (L-15, GIBCO or Flow Labs), supplemented with 20 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2, Sigma), 9% fetal calf serum (Flow Labs) and an antibiotic, gentamycin sulfate (0.1 mg/ml, Sigma)[1,32]. The addition of HEPES was necessary in order to maintain the pH of the air-equilibrated medium. Examination of the fetal calf serum requirement showed that the optimal serum concentration for support of explant attachment to the substratum and neuritic outgrowth was approximately 10%[29]. Goldfish retinal explants could be maintained in culture for 3 weeks or longer without changing the medium.

A collagen substratum was used throughout the major portion of this study, either as the hydrated gel[13] or as a thin film[4]. Collagen was extracted from rat tails by the method of Ehrmann and Gey[12]. A gelatin substratum also supported some neuritic outgrowth, but attachment of the explants to the gelatin was poor. Unless otherwise noted, collagen was the substratum used in these experiments. Polycation-treated glass or plastic surfaces, prepared according to Letourneau[34], (poly-L-lysine, type VI B, Sigma) have recently proven to be suitable substrata for neurite outgrowth from goldfish retinal explants[21].

Preparation of explants from goldfish retina

Retinal explants were prepared as previously described[30]. Eyes were removed

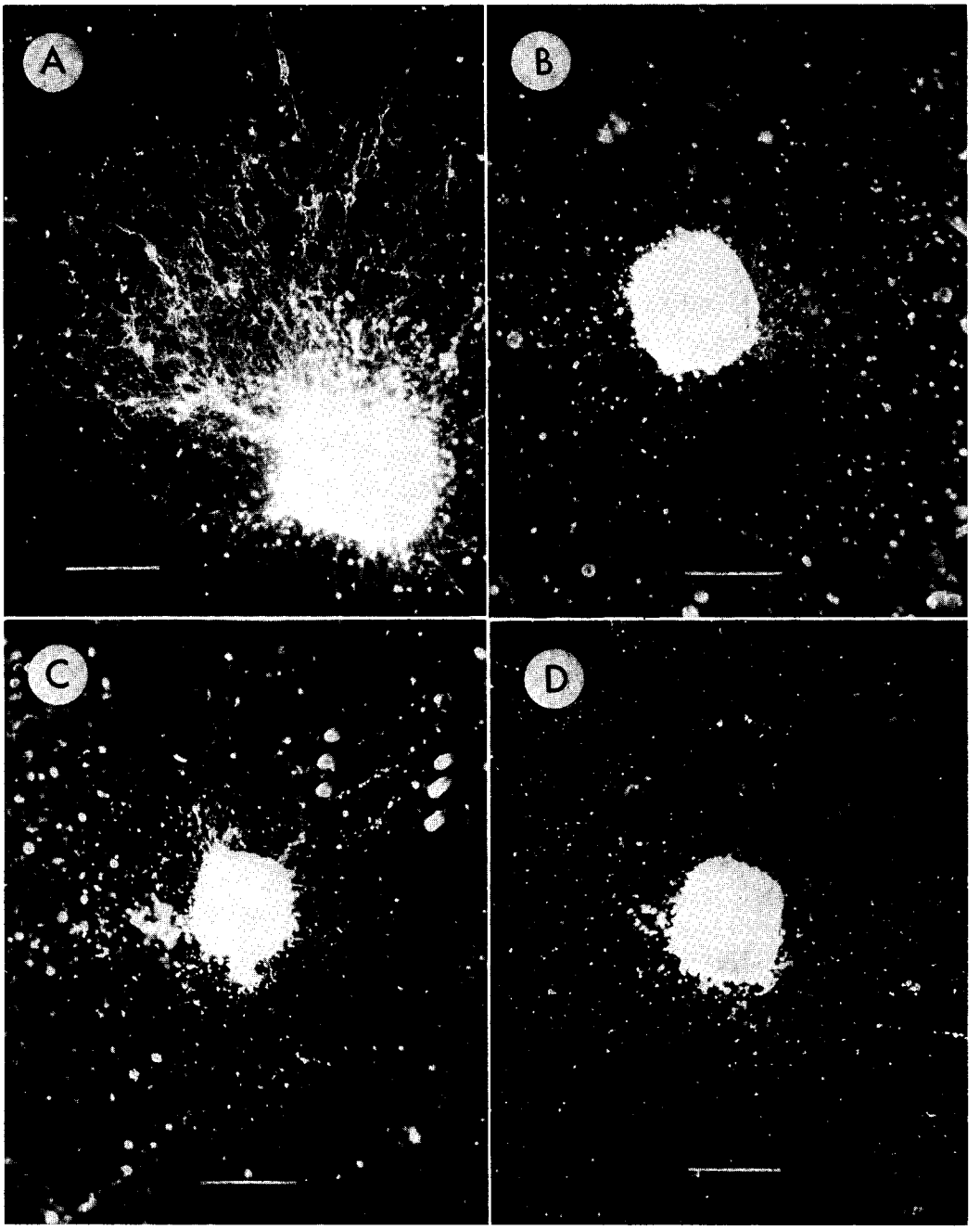

Fig. 1. Neuritic growth densities on collagen gels. Dark-field micrographs of explants with growth typical of each density score. These are representative examples from an atlas compiled for comparison of neuritic densities in calculating the NGI. Neuritic growth with densities similar to A were assigned a score of 4; B, 3; C, 2; and D, 1. Bar = 500 μM.

from dark-adapted fish, then decontaminated with 70% ethanol and rinsed in sterile PBS. The retina was removed from the eye and cut with a McIlwain tissue chopper into 500–600 μm squares. The retinal pieces were transferred through two changes of PBS to remove debris, then placed in a 35 mm tissue culture dish containing approximately 0.5 ml of nutrient medium. The amount of medium in the culture dish was sufficient to cover the explants yet did not allow them to float free in the medium. No attempt was made to orient the explants so that the inner or outer retinal layer was up. Culture dishes normally contained 16–20 explants each and were incubated in humid plastic chambers at room temperature.

Post-crush retinal explants were usually from fish whose right optic nerve had been crushed 10–14 days previously. 'Control' explants were obtained from the left eye

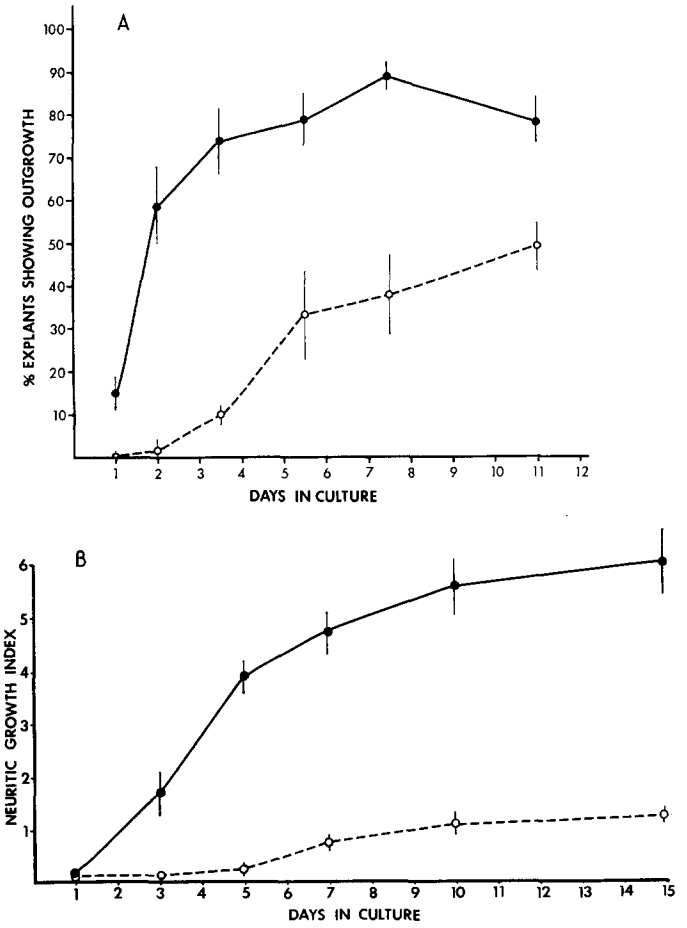

Fig. 2A: effect of prior optic nerve crush on growth of neurites from retinal explants. The fraction of retinal explants exhibiting neurite growth was determined in cultures from control retinas (○, n = 242) or from retinas 10–14 days after optic nerve crush (●, n = 251). Means of 6 experiments ± S.E.M. B: neuritic growth index was a measure of both the density and length of neuritic growth (see text). Retinal explants were from fish whose optic nerve was crushed 11–12 days prior to explantation (●, n = 104) or from the unoperated opposite eye (○, n = 96) ± S.E.M.

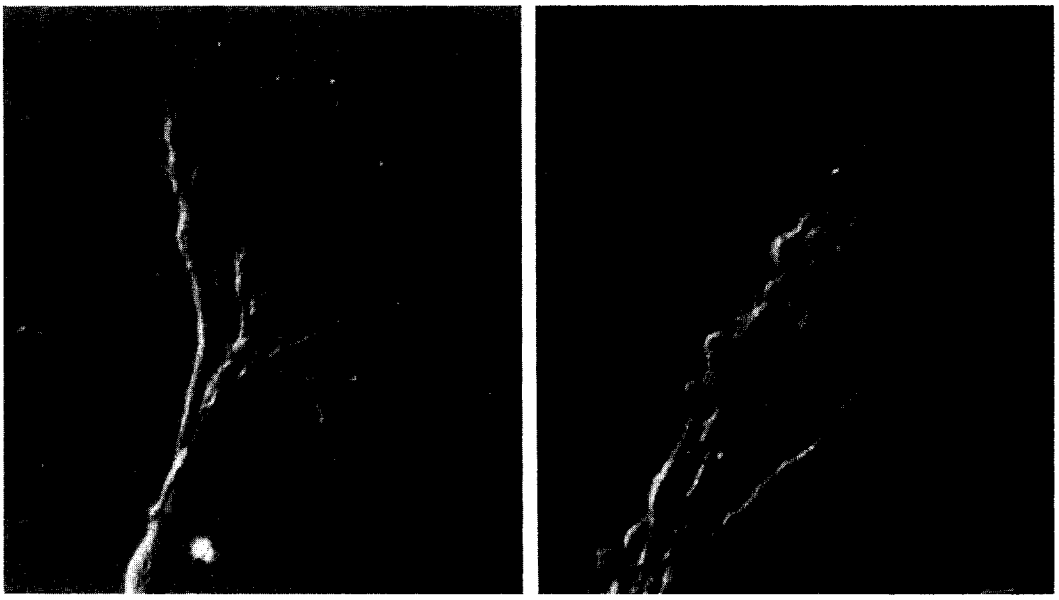

Fig. 3. Interference-contrast micrograph of neuritic growth cones. A: growing tips of two or more intertwined neurites. B: fused growth cone at the end of a large bundle of neurites. Bar = 10 μM.

of the same animal and are referred to as 'control' or 'unoperated'. Retinal explants obtained from untreated fish are termed 'normal'.

Measurement of outgrowth

In initial studies the presence or absence of neurites and of non-neuronal elements was expressed for each dish as a percentage of explants showing neuritic or non-neuronal outgrowth[1]. While introducing some degree of quantitation, the procedure suffered from the obvious disadvantage of not accurately reflecting the amount of growth present, since an explant with a single neurite was given the same score as one with extensive neuritic outgrowth. A new scoring procedure was developed which took into account two aspects of the neuritic growth, fiber density and fiber length, from which a neuritic growth index (NGI) was calculated. This method is similar to that described by Mizell and Bamburg[37]. Neurite density was scored on a scale of 0–4; a score of 0 indicated that no neurites were present at the edge of the explant, while a score of 4 was given for explants with high fiber densities. The length of the fibers was determined with an ocular micrometer, which measured 530 μm under standard observation conditions (10 $\times$ objective, 12.5 $\times$ ocular). A standard zone of outgrowth was thus defined as 530 μm. A score of 0.5 was given to explants with an average fiber length of up to one-half the micrometer scale (265 μm), a score of 1 for 265–530 μm outgrowth; 2 for 530–1060 μm, etc., up to a maximum of 4. To obtain the NGI for each explant, the density score was multiplied by the length score. Individual scores

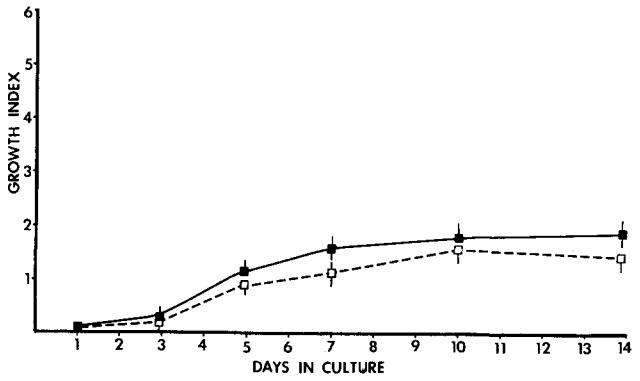

Fig. 4. Effect of prior optic nerve crush on the non-neuronal growth index. Explants were taken from retinas 11–12 days following crush of the optic nerve (■, n = 104) or from control retinas (□, n = 96) ± S.E.M.

from approximately 50 explants were averaged to obtain the NGI for each experimental group.

In order to facilitate the scoring of fiber densities from individual explants, an atlas was assembled with photographs of explants illustrating growth typical of each density score (Fig. 1). Replicate NGI determinations by a single observer differed by less than 8%.

The growth of non-neuronal cells was evaluated by a simple 0–4 grading system. A score of 0 was given to explants with no visible non-neuritic growth at the edge of the explant. Non-neuronal cells generally proliferated only after several days in culture, often forming a monolayer extending up to 1–2 zones from the explant edge. In such cases, the explants were given a score of 4. Intermediate degrees of non-neuronal outgrowth were scored accordingly.

RESULTS

Neuritic growth

We previously reported a striking difference in the growth of control and post-crush retinal cultures[30]. The latter exhibited a vigorous neuritic outgrowth beginning as early as a few hours after explantation. The fraction of explants from post-crush retina with neuritic outgrowth increased dramatically during the first few days in culture, reaching nearly 90% by 7 days (Fig. 2A). In explants of control retina, there was a delayed appearance of neurites, which were observed only after 2–4 days in culture. The fraction of explants exhibiting neurites from the control tissue was always less than that from post-crush retinal explants, although the difference between the two groups diminished with time to approximately 2-fold after 2 weeks in culture. With the neuritic growth index (NGI) as a more sensitive and quantitative measure of explant outgrowth, an even greater difference between control and post-crush retinal cultures becomes apparent (Fig. 2B). As judged by the NGI, after 2 weeks in vitro, the neuritic growth from post-crush retinal explants exceeded that of comparable controls by nearly 6-fold.

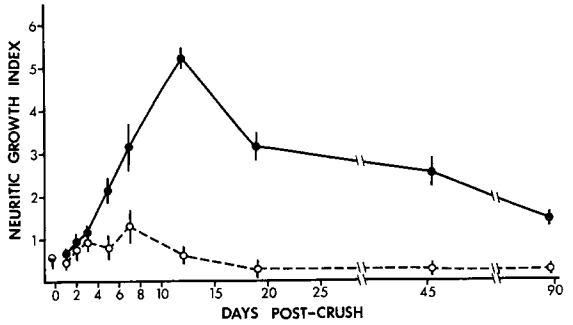

Fig. 5. The effect of the post-crush interval on neuritic growth index of post-crush and normal retinal explants. The interval from crush of the optic nerve to explantation of goldfish retina was varied and the NGI determined on day 7 in culture. Explants were taken from post-crush retina (●) and from the unoperated opposite eye (○). Data from normal (untreated) fish are plotted at 0 time (◒). The 12-day point represents pooled data from fish whose optic nerve had been crushed 11 or 12 days prior to explantation. Individual data points are means of 50 to over 200 explants.

The neurites appeared as thin single fibers or as bundles of fibers. Each characterized by a number of varicose enlargements and terminating in a growth cone with microspikes (Fig. 3).

Non-neuronal cells

Non-neuronal cells migrated onto the substratum from the explants, first appearing after several days in vitro. Explants from post-crush and control retina showed a small, but significant difference in this non-neuronal growth (Fig. 4). Control explants invariably had lower scores. Fibroblast-like cells were the predominant non-neuronal cell types present in these cultures, generally comprising 95–100% of the total non-neuronal cell population. The number of fibroblast-like cells emerging from an individual explant was quite variable. They frequently formed a monolayer about the explant. Pigmented epithelial cells were occasionally observed and easily identified by the presence of prominent granules. Macrophages were infrequently found in the retinal cultures.

Post-crush interval

Experiments were performed to establish at what point following crush of the optic nerve maximal neuritic outgrowth would occur. The enhanced neuritic growth from post-crush retinal explants was compared to that obtained from explants from the unoperated (opposite) eye and from the eyes of untreated normal fish. The post-crush interval is the time between the crush of the optic nerve and explantation of the retina. The NGI shown in Fig. 5 represents the amount of neurite outgrowth present following 7 days in culture, and does not reflect possible variations in delay in initiation of neurites from cultures obtained at the various post-crush intervals. The cultures explanted after short post-crush intervals (1–2 days) in fact did not exhibit neurites until 5 days in vitro. Retinas obtained from fish whose optic nerve had been crushed 3–5 days prior to explantation did not issue neurites before culture day 3,

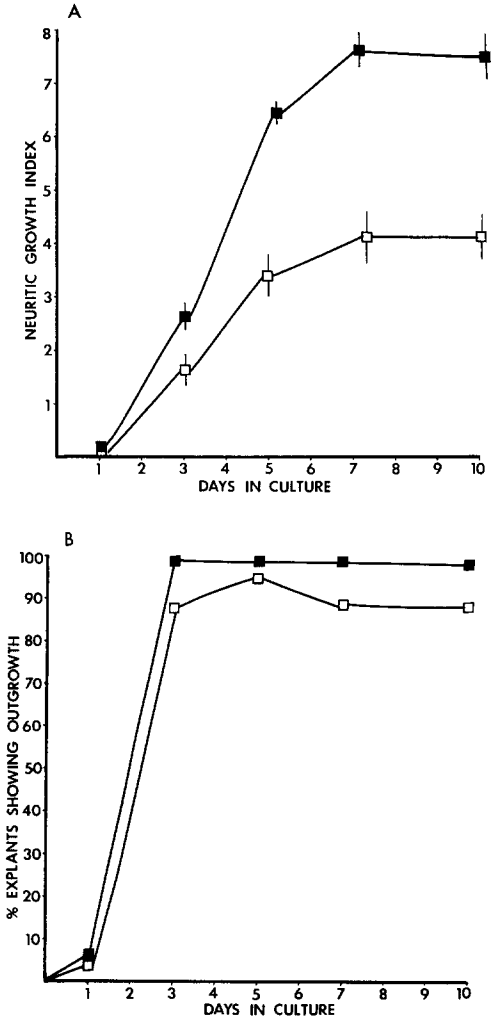

Fig. 6A: neuritic growth from central compared to peripheral retinal explants. Explants were prepared from central (□, n = 94) or peripheral (■, n = 92) retinal regions of post-crush retina and the NGI was measured ± S.E.M. B: the results of an experiment in which the fraction of explants with neuritic growth from central retina were compared to peripheral retinal explants. The fraction of explants exhibiting neuritic growth from central (□, n = 94) and peripheral. (■, n = 92) regions of post-crush retina was determined after the indicated periods in culture.

while 7 or 12 day post-crush explants generally first grew neurites within 24 h following explantation. There was no clear difference in the neuritic growth from post-crush and control cultures until explantation 5 days following optic nerve crush. The difference increased rapidly, reaching a maximum in the interval 11–12 days post-crush. The 12-day point represents pooled data from that period. The NGI values progressively diminished as the post-crush explant interval was lengthened, yet even after 89 days a measurable difference was still present.

Regional differences in growth of goldfish retina in culture

The goldfish retina grows throughout life by adding new cells from a germinal zone at the ciliary margin. Thus, chronologically 'older' cells lie progressively closer to the optic disc[25]. It was of interest to compare the growth of cultures obtained from peripheral retina to those of more central regions, since regenerative responses to axotomy are more vigorous in the young (but not neonatal) animal compared to the adult[35]. There was considerably more growth from peripheral than from central explants of post-crush retina (Fig. 6A). Since all regions of the retina were undergoing a regenerative response, the difference between peripheral and central explants is not as apparent when calculated as percentage of explants showing outgrowth (Fig. 6B).

Histology of retinal explants

Histological examination of post-crush retinal explants has revealed a progressive degeneration of the cells, beginning with the photoreceptors and followed by the inner nuclear layer[26,29]. The ganglion cells alone survived. These studies were extended to control retinal explants to determine if the control ganglion cells develop the characteristic cellular hypertrophy and basophilia associated with axonal regeneration in vivo[42].

Retinal explants were examined by histologic section through the retinal thickness 1, 3, 5, 8 and 12 days following explantation onto collagen gels. Ganglion cell diameters were measured in both post-crush and control explants. Ganglion cells in post-crush cultures did not increase in size any further with time in vitro (Fig. 7). Ganglion cells in control retinal explants demonstrated a nearly linear increase in

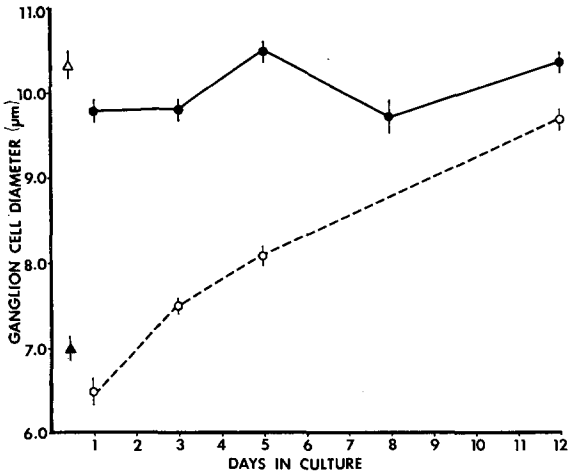

Fig. 7. Ganglion cell diameters were measured with an ocular micrometer in fish whose right optic nerve had been crushed in situ 12 days prior to sacrifice. The diameter of the ganglion cells in the left eye (△) was greater than in the unaffected right eye (▲). Retinal explants taken from fish 12 days after optic nerve section were grown on collagen gels. The ganglion cell diameters from the post-crush explants (●) were compared to those from control cultures (○). At least 200 cells were measured at each point (± S.E.M.).

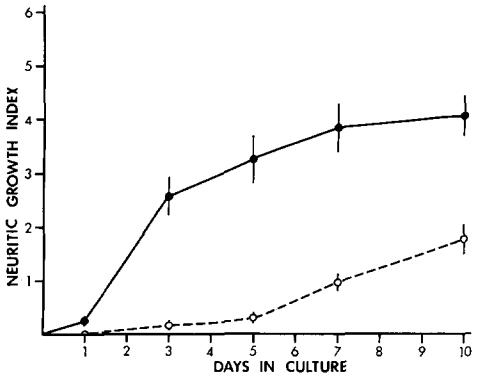

Fig. 8. Neuritic growth from explants of hemiaxotomized retina. The right caudal tectum was removed from goldfish and 7 days later the retina was removed and divided into nasal and temporal hemiretinas. Explants from each group were cultured in the same or different culture dishes. The NGI of nasal (●, axotomized, n = 56) and temporal (○, control, n = 51) explants was determined after the indicated periods in vitro. ± S.EM.

cellular diameter with increasing time in culture. After 12 days in vitro, they were comparable in size to post-crush ganglion cells either in vivo or in vitro. This hypertrophy was accompanied by nuclear eccentricity and increased cytoplasmic basophilia, similar to morphological changes in these cells following optic nerve section in vivo[42].

Hemiaxotomized retina

The following experiments were designed to test whether the retina's ability to regenerate optic fibers following section is the result of a general activation of all of the ganglion cells in the retina or is restricted to only those ganglion cells whose axons have been severed. These studies were prompted by earlier reports in the literature of morphological and biochemical changes following axotomy in related, but unaffected structures[3,17,19,28,44]. In the goldfish, it is possible to selectively cut the optic fibers from restricted regions of the retina by ablation of specified areas of the optic tectum. Thus, within the same organ, a fraction of the ganglion cells have been axotomized while the others are left intact.

The nasal half of the left retina was axotomized by ablation of the caudal half of the right optic tectum. Twelve days following surgery, the ganglion cells in the nasal hemiretina had become hypertrophied while those in the temporal hemiretina appeared normal. Explants were prepared from nasal and temporal hemiretina 7–12 days following surgery and cultured on collagen gels in the same or separate dishes. Nasal (axotomized) retinal cultures exhibited vigorous neuritic outgrowth while temporal retinal explants grew neurites poorly (Fig. 8). The growth of the temporal retinal explants was not improved by culturing in the same dish with nasal explants. The difference in neuritic growth from these tissues was thus similar to that observed when post-crush retinal explants were compared with those from the unoperated opposite eye (Fig. 2). While the data shown in Fig. 8 were from retina explanted 7 days

following surgery, similar results were obtained from cultures explanted after 12 post-operative days. There was no difference between the two groups with respect to numbers of non-neuronal cells. The results indicate that axotomy stimulates only those ganglion cells whose fibers have been severed and has no measurable effect on neighboring intact ganglion cells.

DISCUSSION

The maintenance and growth of goldfish retina in explant culture is rather simple, largely due to the ability of the poikilothermic tissue to grow at room temperature in an air-equilibrated nutrient medium and in the presence of an effective antibiotic. Preparation of the explants is straightforward. The retina and other ocular structures are enclosed in a thick scleral covering making it possible to sterilize the exterior of the eye without damage to the interior tissues. The use of a mechanical tissue chopper to prepare retina explants of uniform size is a further convenience. Champy[7,8] long ago pointed out the advantage of using retina as an organ culture preparation, noting that it is essentially a tissue 'slice' of uniform thickness. The goldfish retina is avascular, having no penetrating blood vessels; its cells ordinarily receive oxygen and other metabolites by diffusion from vessels in the choroid and those lying on the vitreal surface, a situation not unlike that produced in the culture dish.

It is generally acknowledged that most mature neurons fail to survive in culture[36], and most favorable results in brain tissue culture are obtained with embryonic tissue explanted at a stage when morphogenesis is nearly complete and differentiation is beginning[43]. The culture of the adult goldfish retina is thus one of a few examples of survival of adult nervous tissue in vitro. Adult amphibian ganglia[22,45] and teleost CNS tissue[10] have recently been found to survive and grow in culture.

The ability of the goldfish retina to grow neurites in culture correlates well with the response of the retinal ganglion cell to axotomy. Histological studies of Grafstein and Murray[38,39,41,42] have shown that the morphological changes and enhanced labeling of protein and RNA can be observed in the goldfish visual system during the first week following axotomy. An increase in axonal transport[15,16] was also observed at this time. It is of particular interest that the labeling of the microtubular subunit protein, tubulin, is selectively enhanced within 5 days following optic nerve section[20], and increases in nucleoside uptake, ribosomal and poly(A)-RNA labeling are seen[5,6]. The enhancement of neuritic growth due to section of the optic nerve is first demonstrable at 5 days following nerve crush and is maximal at 12 days.

On histological examination of the retinal explants it was seen that the ganglion cells survived much better in culture than other neuronal cell types. If we consider that adult tissues in general do not survive well in culture, the degeneration seen in most of the retinal elements is to be expected, and the anomalous phenomenon is that the ganglion cells survive. It may well be that the axotomized ganglion cell shares with developing cells an enhanced biosynthetic capability which is responsible for both the neuritic outgrowth and its selective survival in retinal explant cultures[18,43,46]. The

morphological response of the explanted control retina, parallels in several ways the alterations seen in vivo following optic nerve crush. For example, after a few days in culture the control retina exhibits hypertrophy of the ganglion cell body, basophilia and some neuritic outgrowth. The delay in outgrowth of neurites following explantation of the control retina corresponds to some extent to the time course of the effect of crush on subsequent outgrowth from explanted post-crush retina. Control retinal explants first issue neurites on or about day 5 in vitro. However, even after two or three weeks in culture, the amount of neuritic outgrowth from these explants does not approach that from post-crush retinal explants.

The poor growth of the control explants may reflect the failure of the goldfish retinal explants to survive more than a few weeks in culture. This in turn may be related to the adult nature of this tissue. The growth of neurites from post-crush retinal cultures occurs largely during the first week in culture. Only modest increases in NGI occur in the subsequent week, with death ensuing after the third week in culture. This suggests the possibility that the ganglion cells in the explant have sufficient stores of endogenous nutrients or preformed structural elements[20], to sustain growth for only a few days in vitro following which synthetic processes can no longer support vigorous neuritic extension. It would follow that by the time the cellular machinery required for axonal regrowth was mobilized in the control retinal explant, unfavorable conditions imposed by explantation would become limiting, resulting in poor neurite outgrowth. Observation of neuritic growth from larval Xenopus retinal cultures support this view[1], since the unoperated normal eye, after a lag period, generally has as much neuritic growth after two weeks in culture as the post-crush retinal cultures. This suggests that the larval ganglion cells survive and are capable of vigorous neuritic outgrowth even after long periods in vitro.

Our initial observations suggested that, at early times post-crush, neuritic growth from the retina of the opposite (unoperated) eye was greater than that of retinal cultures taken from normal, untreated fish (Fig. 5). With further experiments, this difference proved not to be consistently reproducible. Nevertheless, this hint of an effect suggested a possible role for systemic or diffusible factors in the regenerating goldfish visual system. In a variety of paired nervous structures, axotomy or trauma to one side produces metabolic and morphologic changes in the symmetric contralateral structure. Perhaps the best known example of this is sympathetic ophthalmoplegia[47]. A penetrating wound to the eye can result in inflammation and loss of the opposite uninvolved eye 1–3 months following the initial injury. The mechanism by which this occurs is not understood, but the data suggests the intervention of humoral factors, presumably immunologic. Greenman[17] reported that section of peroneal nerve on one side resulted in morphological changes in both the severed nerve and its contralateral counterpart, in which a decrease in mean fiber diameter was seen. Nittono[44] found that if a peripheral branch of the right trigeminal nerve was cut, degenerating and regenerating fibers were found on the left side. Austin and co-workers[3,28] found that following axotomy of the nodose ganglion, there were increases in acid phosphatase and carbamoyl phosphate synthetase activity on the cut side and similar, but smaller changes on the uncut side. Hamberger and Sjöstrand[19] reported similar alterations of

succinate dehydrogenase activities following section of a single hypoglossal nerve. Blood-borne diffusible factors have been suggested to explain these various phenomena although there is no direct evidence for this in the nervous system[9]. The present study adds no support for a role of humoral factors in the regenerative response. The morphological similarity of the ganglion cells in control retinal explants after several days in vitro to post-crush ganglion cells indicates that a systemic or blood-borne factor is not required to elicit the ganglion cell reaction to axotomy. Following half-tectal ablation, the fact that one can find side-by-side regenerating tissue and normal tissue within a single retina argues against the involvement of diffusible factors within the retina itself in the ganglion cell response.

The mechanisms that induce the reinitiation of axonal growth following axotomy can be approached in tissue culture in a manner not practicable with in vivo studies. The primary intent of these studies was to develop an in vitro model for the study of nerve growth, particularly as it applies to regeneration in the central nervous system. The usefulness of the goldfish retinal explants as such a model is confirmed by the observed correlation between morphological changes in the retinal ganglion cells and neurite outgrowth. The rapid appearance of neurites from the post-crush retina in culture is direct evidence of the axonal regrowth which was occurring in the animal at the time of explantation. The vigorous growth of neurites from post-crush retina thus offers a unique opportunity to examine in vitro fibers whose behavior has been well-studied in vivo.

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REFERENCES

1 Agranoff, B. W., Field, P. and Gaze, R., Neurite outgrowth from explanted Xenopus retina: an effect of prior optic nerve section, *Brain Research*, 113 (1976) 225–234.
2 Attardi, D. and Sperry, R., Preferential selection of central pathways by regenerating optic fibers, *Exp. Neurol.*, 7 (1963) 46–64.
3 Austin, L., Kaye, P. and Gunning, P., Changes in RNA metabolism in response to axotomy. In *Int. Soc. Neurochem., 5th meeting*, Barcelona, 1975, 214.
4 Bornstein, M., Reconstituted rat-tail collagen used as substrate for tissue culture on coverslips in Maximow slides and roller tubes, *Lab. Invest.*, 7 (1958) 134–137.
5 Burrell, H., Dokas, L. and Agranoff, B. W., Poly(A)-RNA in goldfish retina and optic nerve regeneration, *Fed. Proc.*, 36 (1977) 802.
6 Burrell, H., Dokas, L. and Agranoff, B. W., RNA metabolism and optic nerve regeneration in the goldfish, *J. Neurochem.*, 31 (1978) 289–298.
7 Champy, C., La presence d'un tissu antagoniste maintient la differenciation d'un tissu cultive en dehors de l'organisme, *C.R. Soc. Biol. (Paris)*, 76 (1913) 31–32.

8 Champy, C., Quelques resultats de la method de culture des tissues. IV. La retine, *Arch. Zool. Exp. Gen.*, 55 (1914) 1–18.
9 Cragg, B., What is the signal for chromatolysis?, *Brain Research*, 23 (1970) 1–21.
10 Deboni, U., Seger, M., Scott, J. and Crapper, D., Neuron culture from adult goldfish, *J. Neurobiol.*, 7 (1976) 495–512.
11 Dulbecco, R., Plaque formation and isolation of pure lines with poliomyelitis viruses, *J. exp. Med.*, 99 (1954) 167–199.
12 Ehrmann, R. and Gey, G., The growth of cells on a transparent gel of reconstituted rat tail collagen, *J. nat. Cancer Inst.*, 16 (1956) 1375–1392.
13 Elsdale, T. and Bard, J., Collagen substrata for studies on cell behavior, *J. Cell Biol.*, 54 (1972) 626–637.
14 Gaze, R. M., *The Formation of Nerve Connections*, Academic Press, London, 1970.
15 Grafstein, B., Role of slow axonal transport in nerve regeneration, *Acta neuropath. (Berl.)*, Suppl. V (1971) 144–152.
16 Grafstein, B. and Murray, M., Transport of protein in goldfish optic nerve during regeneration, *Exp. Neurol.*, 25 (1969) 494–508.
17 Greenman, M., Studies on the regeneration of the peroneal nerve of the albino rat: number and sectional areas of fibers: area relation of axis to sheath, *J. comp. Neurol.*, 23 (1913) 479–513.
18 Griffith, A. and Lavelle, A., Development of protein changes in normal and chromatolytic facial nerve nuclear regions, *Exp. Neurol.*, 33 (1971) 360–371.
19 Hamberger, A. and Sjöstrand, J., Respiratory enzyme activities in neurons and glial cells of the hypoglossal nucleus during nerve regeneration, *Acta physiol. scand.*, 67 (1966) 76–88.
20 Heacock, A. and Agranoff, B. W., Enhanced labeling of a retinal protein during regeneration of optic nerve in goldfish, *Proc. Nat. Acad. Sci. (Wash.)*, 73 (1976) 828–832.
21 Heacock, A. and Agranoff, B. W., Clockwise growth of neurites from retinal explants, *Science*, 198 (1977) 64–66.
22 Hill, C. and Burnstock, G., Amphibian sympathetic ganglia in tissue culture, *Cell Tiss. Res.*, 162 (1976) 209–233.
23 Jacobson, M., *Developmental Neurobiology*, Holt, Rinehart and Winston, New York, 1970.
24 Jacobson, M. and Gaze, R., Selection of appropriate tectal connections by regenerating optic nerve fibers in adult goldfish, *Exp. Neurol.*, 13 (1965) 418–430.
25 Johns, P. R., Growth of the adult goldfish eye. III. Source of the new retinal cells, *J. comp. Neurol.*, 176 (1977) 343–357.
26 Johns, P. R., Heacock, A. M. and Agranoff, B. W., Neurites in explant cultures of adult goldfish retina derived from ganglion cells, *Brain Research*, 142 (1978) 531–537.
27 Johns, P. R., Yoon, M. G. and Agranoff, B. W., Directed outgrowth of optic fibers regenerating in vitro, *Nature (Lond.)*, 271 (1978) 360–362.
28 Kaye, P. and Austin, L., Neural metabolic responses to axon damage, *Proc. Austr. Biochem. Soc.*, 8 (1975) 118.
29 Landreth, G. E., *Explant Culture of the Adult Goldfish Retina*, Ph. D. Thesis, University of Michigan, 1977.
30 Landreth, G. E. and Agranoff, B. W., Explant culture of adult goldfish retina: effect of prior optic nerve crush, *Brain Research*, 118 (1976) 299–303.
31 Landreth, G. E. and Agranoff, B. W., Explant culture of the adult goldfish retina: a model for CNS regeneration, *Neurosci. Abstr.*, 2 (1976) 1025.
32 Laskey, R., The use of antibiotics in the preparation of amphibian cell cultures from highly contaminated material, *J. Cell Sci.*, 7 (1970) 653–659.
33 Leibovitz, A., The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere, *Amer. J. Hyg.*, 78 (1963) 173–180.
34 Letourneau, P., Possible roles for cell-to-substratum adhesion in neuronal morphogenesis, *Develop. Biol.*, 44 (1975) 77–91.
35 Lieberman,. A., Some factors affecting retrograde neuronal responses to axonal lesions. In R. Bellairs and E. Gray (Eds.), *Essays on the Nervous System*, Oxford Press, London, 1974, pp. 70–105.
36 Lumsden, C., Nervous tissue in culture. In G. Bourne (Ed.), *The Structure and Function of Nervous Tissue*, Academic Press, New York, 1968, pp. 68–138.
37 Mizell, S. and Bamburg, J., Studies on the action of nerve growth factor. I. Characterization of a simplified in vitro culture system for dorsal root and sympathetic ganglia, *Develop. Biol.*, 49 (1976) 11–19.

38 Murray, M., ^{3}H-Uridine incorporation by regenerating retinal ganglion cells of goldfish, *Exp. Neurol.*, 39 (1973) 489–497.
39 Murray, M., Regeneration of retinal axons into goldfish optic tectum, *J. comp. Neurol.*, 168 (1976) 175–196.
40 Murray, M., Delayed regeneration of the retino-diencephalic projections in the goldfish *Carassius auratus*, *Brain Research*, 125 (1977) 149–153.
41 Murray, M. and Forman, D., Fine structural changes in goldfish retinal ganglion cells during axonal regeneration, *Brain Research*, 32 (1971) 287–298.
42 Murray, M. and Grafstein, B., Changes in morphology and amino acid incorporation of regenerating goldfish optic neurons, *Exp. Neurol.*, 23 (1969) 544–560.
43 Murray, M. R., Nervous tissues isolated in culture. In A. Lajtha (Ed.), *Handbook of Neurochemistry, Vol. 5A*, Plenum Press, New York, 1971, pp. 393–438.
44 Nittono, K., On bilateral effects from the unilateral section of branches of the nervus trigeminus in the albino rat, *J. comp. Neurol.*, 35 (1935) 133–161.
45 Padjen, A., Siggins, G. and Forman, D., Long-term growth and electrophysiological properties of peripheral ganglia of adult frog in vitro, *Neurosci. Abstr.*, 1 (1975) 813.
46 Price, D. L. and Porter, K., The response of ventral horn neurons to axonal transection, *J. Cell Biol.*, 53 (1972) 24–37.
47 Sorsby, A., *Modern Ophthalmology, Vol. 3*, 2nd ed., Lippincott, Philadelphia, 1972, pp. 485–493.
48 Springer, A. and Agranoff, B. W., Effect of temperature on rate of goldfish optic nerve regeneration: a radioautographic and behavioral study, *Brain Research*, 128 (1977) 405–415.
49 Springer, A. D. and Landreth, G. E., Direct ipsilateral retinal projections in goldfish, *Brain Research*, 124 (1977) 533–537.