Brain Research, 142 (1978) 531–537
© Elsevier/North-Holland Biomedical Press

Short Communications

Neurites in explant cultures of adult goldfish retina derived from ganglion cells

PAMELA RAYMOND JOHNS, ANNE M. HEACOCK and BERNARD W. AGRANOFF

The University of Michigan, Neurosciences Laboratory Building, Ann Arbor, Mich. 48109 (U.S.A.)

(Accepted September 14th, 1977)

We recently described a technique for obtaining neurite outgrowth from explants of larval *Xenopus*[1] and adult goldfish retina[7]. As is true of explants from other neural tissues, such as dorsal root ganglion[3] and spinal cord[12,13], these cultures are comprised of a heterogeneous population of neurons, any of which might give rise to neuritic processes. There was inferential evidence that the neurites in the retinal explants were derived from ganglion cells: they are the only retinal neurons with long, extrinsically projecting axons in vivo, whereas all of the other neuronal types are characterized by short processes confined to the retina. In addition, optic nerve crush prior to explantation stimulates neurite production[1,7]. This finding further implicates the retinal ganglion cells, since they are then in the process of regenerating their severed axons, and are thus already 'primed' for growth at the time of retinal explantation. Since we wished to pursue further studies using the retinal explant system as a model for optic nerve regeneration, it was essential to establish the identity of the neurites definitively.

In the present study we have employed the folowing approaches to determine the source of the neurites: (1) an examination of the histological integrity of the explants, relying on the laminar structure of the retina as an aid in identifying cell types; (2) reduced silver staining to visualize the neurons and their processes; (3) scanning electron microscopic (SEM) observations; and (4) tracing the neurites back to their cells of origin, using the horseradish peroxidase (HRP) technique.

Retinal explants from adult goldfish *(Carassius auratus)*, 6–7 cm body length, were prepared as described previously[7]. In some cases, 5-fluorodeoxyuridine (Sigma, 10^{-4} *M*) was added to the medium to inhibit non-neuronal cell proliferation[4]. The substrata used were glass coverslips (Corning, 22 mm) or plastic tissue culture dishes (Nunclon, 35 × 10 mm) coated with either a collagen film[2] or with poly-L-lysine[4,9]. Ten to 14 days following optic nerve crush, retinas were removed and cut through their full thickness (approximately 150 μm) into 100 μm wide strips, or, alternatively, into 500 μm squares by means of a McIlwain chopper as described previously[7]. The square pieces were placed on the substratum with the vitreal or photoreceptor surface up, whereas the strips were oriented with the cut surface apposed to the sub-

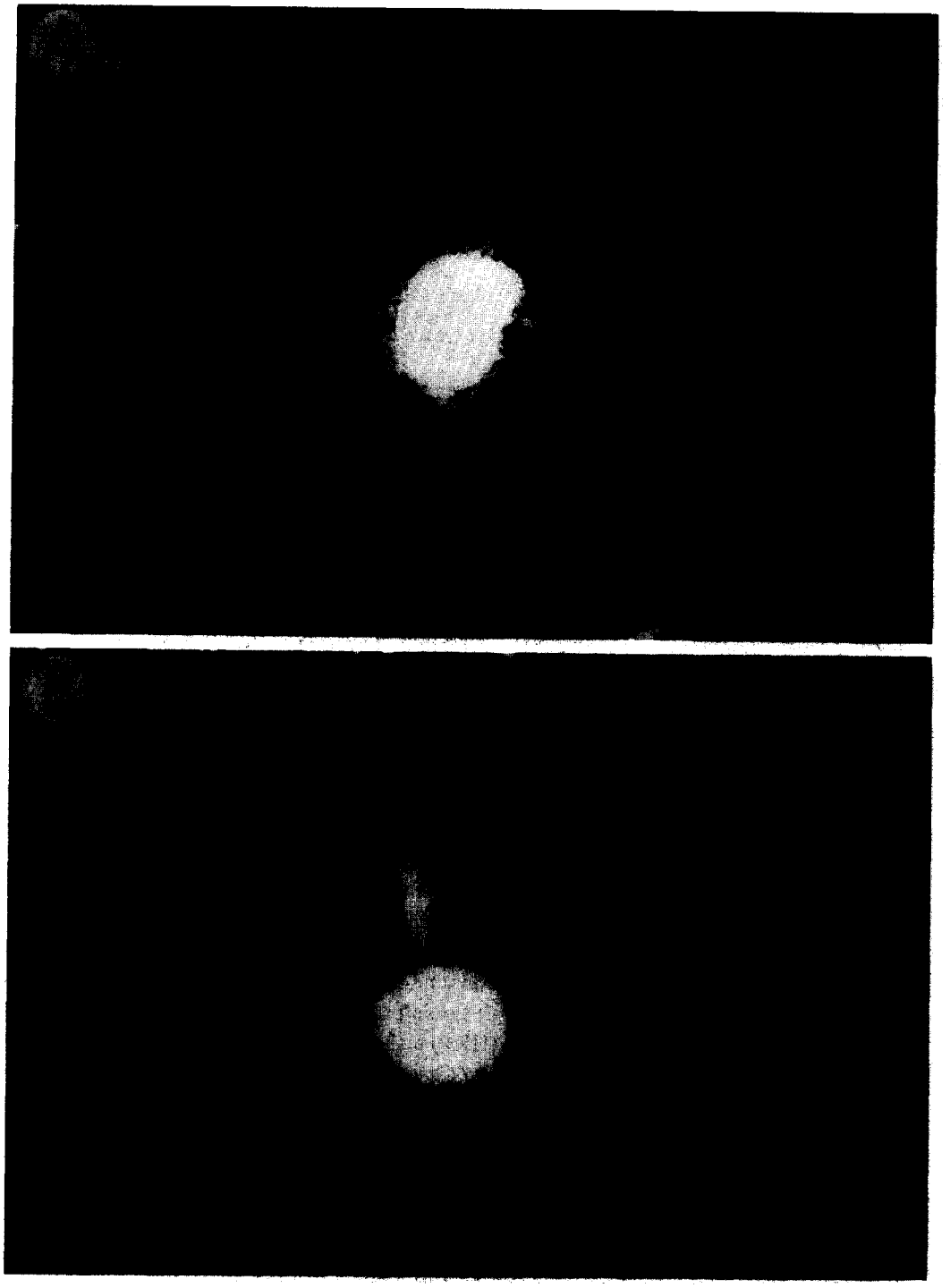

Fig. 1. Retinal explants. Dark-field photomicrographs of living cultures after 8 days (A) and 21 days (B) in vitro. Calibration bar: 1 mm.

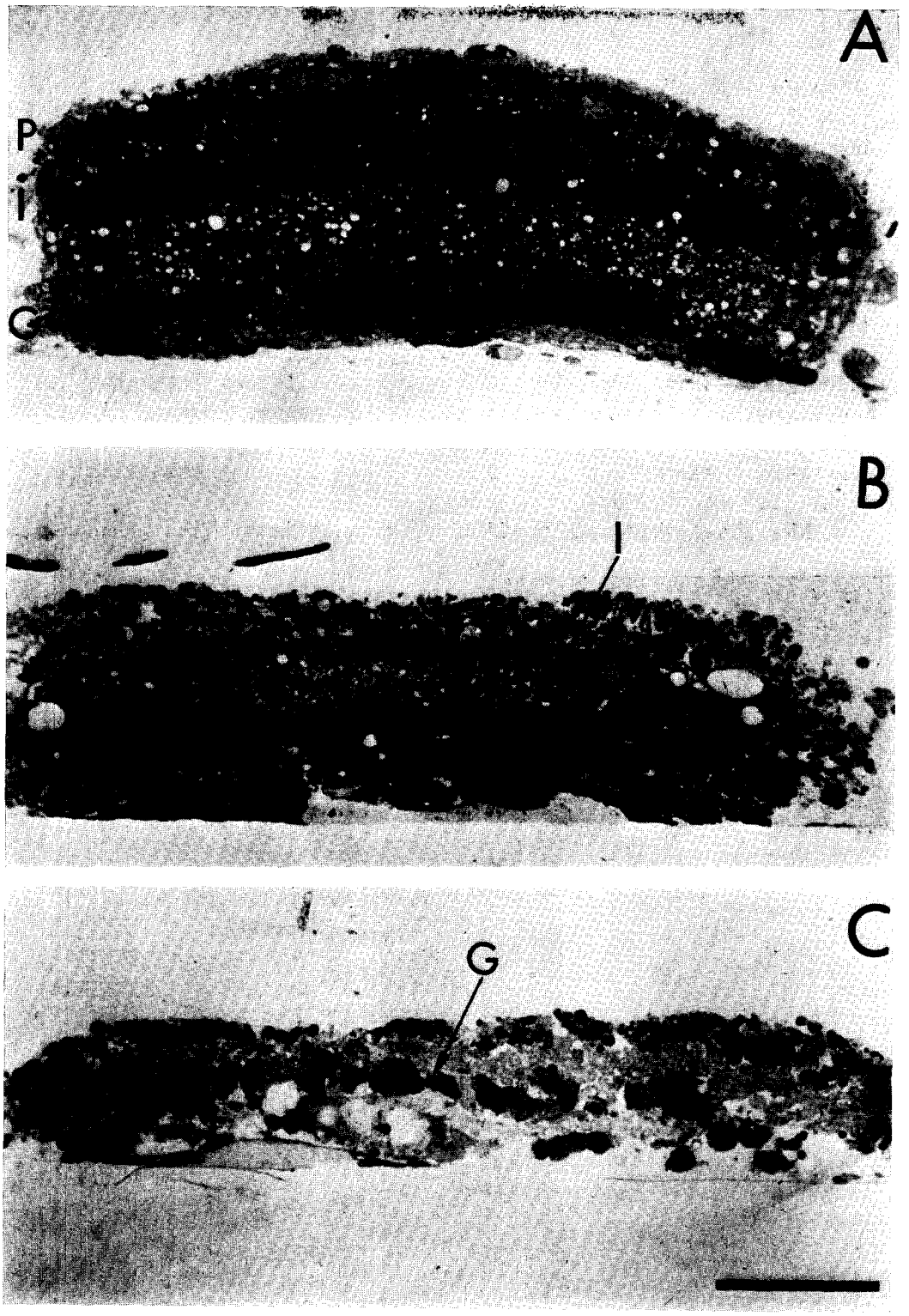

Fig. 2. Histology of retinal explants. Explants grown vitreal side down on plastic dishes were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4, embedded in an Epon-Araldite mixture, sectioned transversely at 1–2 μm and stained with toluidine blue. A: 6 days after explantation. The photoreceptor nuclei (P) are degenerating. B: 14 days. The inner nuclear layer (I) shows signs of degeneration. C: 20 days. The only neurons remaining are the ganglion cells (G) .Calibration bar: 100 μm.

stratum, thus revealing the retinal layers. The two orientations permitted us two, perpendicular views of the retinal tissue: one superficial (viewed en face) and the other internal (seen in cross-section).

The explants grown directly on plastic dishes were sectioned for histological examination. During the first two weeks in vitro, while the neurites were growing out onto the substratum[6,7] (Fig. 1A), the explants showed a progressive, layer-by-layer degeneration of cells, beginning with the photoreceptors (Fig. 2A) and followed by the cells in the inner nuclear layer[6] (Fig. 2B). The ganglion cells alone survived, so that by the end of 3–4 weeks they were the only viable cells remaining (Fig. 2C). Their cytological appearance was not distinguishable from that seen in conventional sections of freshly exised retina folowing optic nerve crush. It should be noted that crushing the goldfish optic nerve results in an axonal reaction in the ganglion cell soma[10,11], and this altered morphology is maintained in culture[6,7] for several weeks. Since the neurites also survive at least that long (Fig. 1B), these histological findings provide indirect evidence that they originate from the ganglion cells.

More direct evidence was provided by preparations in which cell bodies and

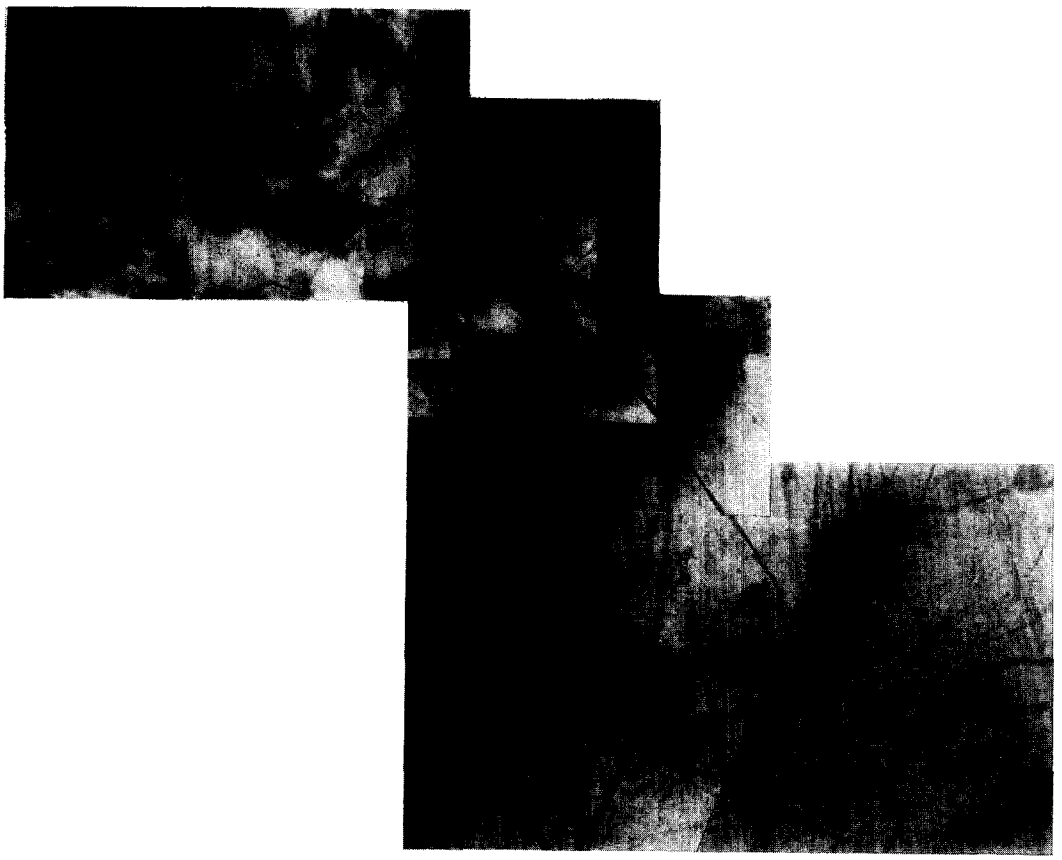

Fig. 3. Silver stained retinal explants. A neurite can be traced (arrows) to a ganglion cell soma. Calibration bar: 10 μm.

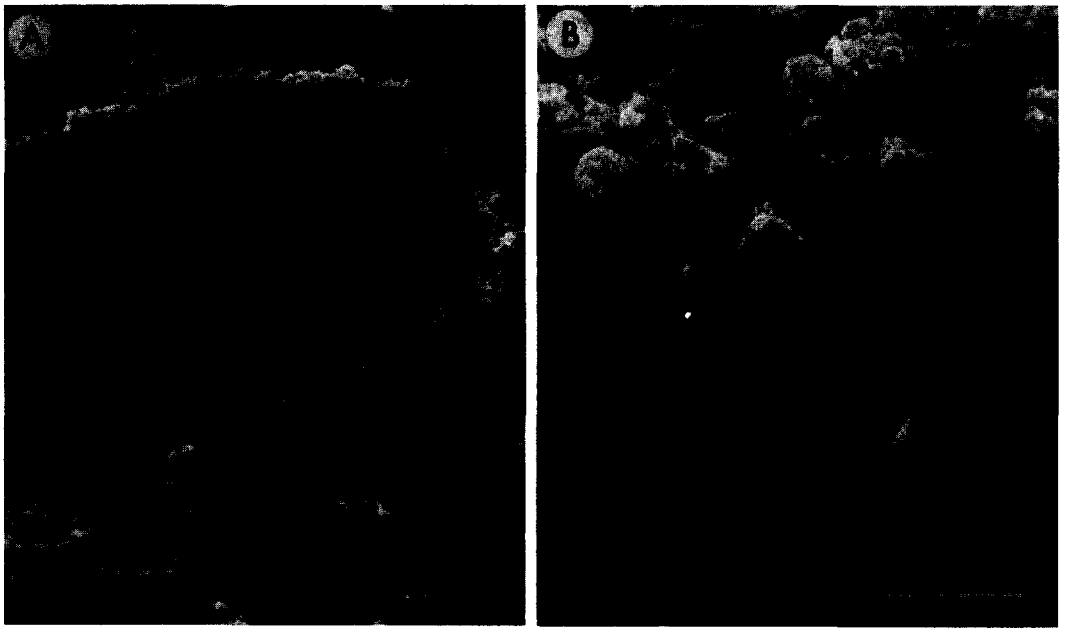

Fig. 4. Scanning electron microscopy of retinal explants. Explants grown on coverslips were fixed as described in Fig. 2, dehydrated, critical point-dried, gold-coated and examined at 15 kV with a JEOL microscope (JSM-U3). The explants were grown so that the retinal layers were viewed in cross section. ROS, rod outer segments; other abbreviations as in Fig. 2. A: in this preparation the laminar structure of the retina was well-preserved, but the neurites were broken off at the edges of the explant as it pulled away from the coverslip. This was a typical artifact of the drying process; only in exceptionally thin explants (in which the retinal laminae were not as well-preserved) did the neurites remain attached to their cells of origin. Calibration bar: 50 μm. B: a neurite (arrow) arising from a cell in the ganglion cell layer. Calibration bar: 10 μm.

processes were stained with Holmes silver nitrate[14]. In favorable instances, individual neurites could be traced to their cells of origin, the perikarya of which invariably had the cytological features of ganglion cells and were located in the ganglion cell layer (Fig. 3). Observations of the cultures using S.E.M. also supported this conclusion. In the example shown in Fig. 4, a neurite is seen to arise from a probable ganglion cell.

Further, and perhaps most definitive, proof of the identity of the neurites was provided by the technique of axonal tracing using HRP[8]. The major difficulty in applying this method to the culture preparation was the rapid diffusion of the HRP through the liquid medium from the site of application near the tips of the neurites, resulting in non-specific labeling of all of the cells in the explant. The problem was solved by covering the explants with a layer of gelatin, in order to impede diffusion. Application of HRP near the cut tips of the neurites resulted in a diffuse and heavy labeling of the fibers[8], as can be seen in Fig. 5A. The only perikarya in the explant which were similarly labeled were of the ganglion cells, and labeled neurites could be traced back to labeled ganglion cell somas (Fig. 5B).

We have thus demonstrated that the neuritic outgrowth from explant cultures of adult goldfish retina arises from ganglion cells. The stimulatory effect of optic nerve

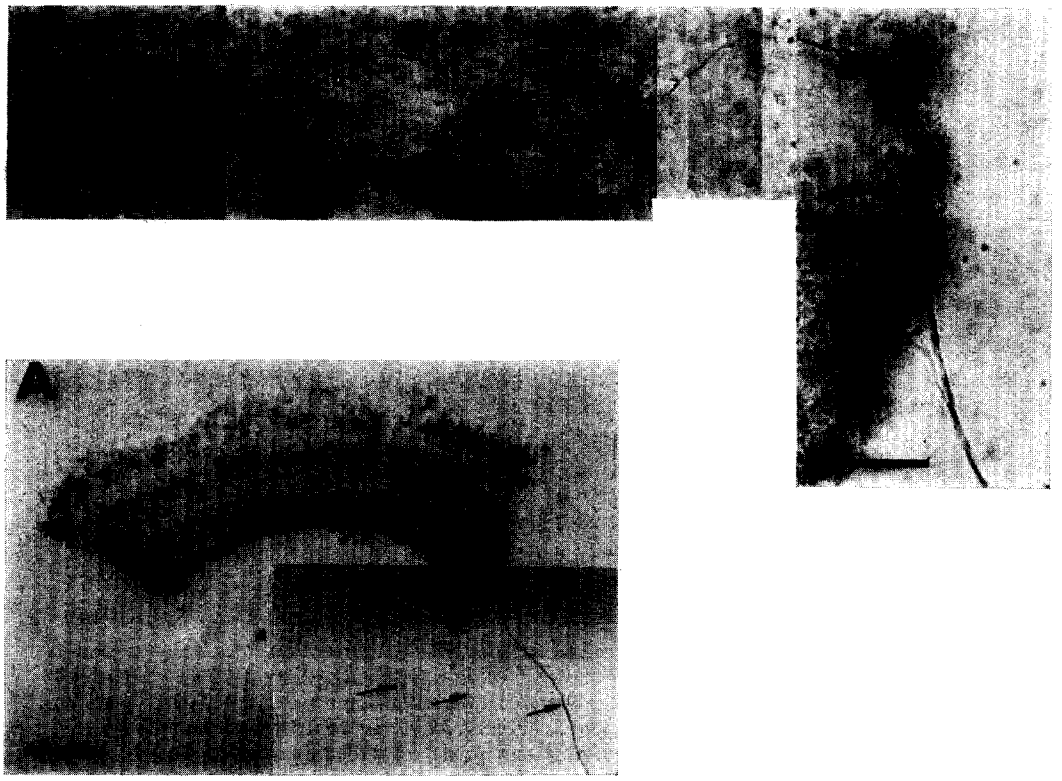

Fig. 5. Neurites labeled with HRP. Explants grown on coverslips were covered with a 4 mm thick layer of 10% gelatin in 0.9% saline. A microliter syringe with a 30-gauge fixed needle was used to inject 0.1 μl of a solution of HRP (Sigma type VI, 100 mg/ml) in phosphate-buffered saline (PBS, pH 7.2). The HRP was applied to neurites cut with the needle near their tips. After 4 h the gelatin was liquified by warming to 25 °C, the coverslip rinsed with PBS, the explants fixed for 2 h at 4 °C in 2% glutaraldehyde and then washed overnight in PBS plus 30% sucrose. The explants were pretreated with diaminobenzidine (DAB, Sigma, 30 mg/100 ml) in PBS, for 15–20 min, and placed in a fresh DAB solution supplemented with 0.002% hydrogen peroxide for an additional 15–20 min. A: retinal explant viewed in cross-section. Neurites (arrow) and ganglion cell somas (G) are filled with HRP. Calibration bar: 100 μm. B: this is the same explant at higher magnification showing a labeled neurite emerging from a labeled ganglion cell (arrow). Calibration bar: 25 μm.

crush suggests that the neurites are axonal rather than dendritic processes. This conclusion is supported by the further observation that the pattern and directionality of the neuritic growth partially mimics that of the optic fibers in vivo[5]. Preliminary studies of the ultrastructure of the neurites are also consistent with their identity as axons. From these observations, taken together, we conclude that the neurites are regenerating optic nerve fibers. The present result underscores the proposal[7] that explant culture of goldfish retina provides a useful in vitro system for further studies of optic nerve regeneration.

This work was supported by NSF BMS 75-03810 and NIH MH 12506-10. P.R. Johns was supported by NIH postdoctoral fellowship NS05518-01. We are grateful

to Dr. T. J. Neary for his suggestion concerning the HRP procedures and to Dr. S. S. Easter for some preliminary transmission electron micrographs. Ms. P. J. Hollingsworth and Mr. D. Webb provided expert technical assistance.

1 Agranoff, B. W., Field, P. and Gaze, R. M., Neuritic outgrowth from explanted *Xenopus* retina: an effect of prior optic nerve section, *Brain Research*, 113 (1976) 225–234.
2 Bornstein, M., Reconstituted rat-tail collagen used as a substrate for tissue culture on coverslips in Maximov slides and roller tubes, *Lab. Invest.*, 7 (1958) 134–137.
3 Chamley, T. H., Mark, G. E., Campbell, G. R. and Burnstock, B., Sympathetic ganglia in culture. I. Neurons, *Z. Zellforsch.*, 135 (1972) 287–314.
4 Heacock, A. M. and Agranoff, B. W., Clockwise growth of neurites from retinal explants, *Science*, 198 (1977) 64–66.
5 Johns, P. R., Yoon, M. G. and Agranoff, B. W., Directed outgrowth of optic fibers regenerating in vitro, in preparation.
6 Landreth, G. E., *Explant Culture of the Adult Goldfish Retina*, Ph.D. Thesis, University of Michigan, 1977.
7 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.
8 LaVail, J. H., The retrograde transport method, *Fed. Proc.*, 34 (1975) 1618–1624.
9 Letourneau, P. C., Possible roles for cell-to-substratum adhesion in neuronal morphogenesis, *Develop. Biol.*, 44 (1975) 77–91.
10 Murray, M. and Forman, D., Fine structural changes in goldfish retinal ganglion cells during axonal regeneration, *Brain Research*, 32 (1971) 287–298.
11 Murray, M. and Grafstein, B., Changes in the morphology and amino acid incorporation of regenerating goldfish optic neurons, *Exp. Neurol.*, 23 (1969) 544–560.
12 Peterson, E. R., Crain, S. M. and Murray, M. R., Differentiation and prolonged maintenance of bioelectrically active spinal cord cultures, *Z. Zellforsch.*, 66 (1965) 130–154.
13 Sobkowicz, H. M., Guillery, R. W. and Bornstein, M. B., Neuronal organization in long term cultures of the spinal cord of the fetal mouse, *J. comp. Neurol.*, 132 (1968) 365–396.
14 Wolf, M. K., Differentiation of neuronal types and synapses in myelinating cultures of mouse cerebellum, *J. Cell Biol.*, 22 (1964) 254–279.