Brain Research, 168 (1979) 628–632

Increased tubulin messenger RNA in the goldfish retina during optic nerve regeneration

H. R. BURRELL, A. M. HEACOCK, R. D. WATER and B. W. AGRANOFF*

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

(Accepted February 1st, 1979)

Axotomy induces profound morphological changes in the neuronal cell body[2,3,10]. Neurons whose axons lie within the central nervous system of higher vertebrates (intrinsic neurons) show little or no functional regenerative capability[4,8]. However, many mammalian neurons whose axons lie outside the CNS, e.g., sensory and motor neurons[5,15] and some intrinsic CNS neurons of lower vertebrates, such as teleosts and amphibia, can regenerate functional axons and re-establish normal synaptic fields[7,9,11]. This recuperative response of the perikaryon to axotomy provides a model for the study of regeneration as well as synaptogenesis and other events thought to underlie neuronal growth, recognition and plasticity.

Regeneration of functional retinotectal fibers following optic nerve crush in goldfish is associated with a number of quantifiable biochemical changes in the retina, including increased nucleoside uptake and phosphorylation[1]. There is also an enhanced labeling of cytoplasmic poly(A)-containing RNA[1] and of the microtubule protein tubulin[6]. The enhanced radioisotopic labeling of goldfish retinal tubulin following optic nerve crush could be attributed either to its increased synthesis or decreased degradation. Since the former explanation could reflect regulation at the transcriptional or translational level and axotomy had resulted in enhanced labeling of poly(A)-containing RNA, we investigated whether retinal poly(A)-RNA obtained following optic nerve crush was enriched in specific messenger RNAs, particularly that for tubulin. Translation of retinal poly(A)-containing RNA in a heterologous, cell-free protein synthesizing system, followed by SDS gel electrophoresis of the products, provided the necessary tools to detect the proposed changes in RNA populations.

Cytoplasmic poly(A)-containing RNA preparations were isolated from both normal and from post-crush retinas, then incubated in a wheat germ protein synthesizing system. The protein labeling patterns from 4, 10 and 15 day post-crush incubations and their controls are shown in Fig. 1. A distinct increase in radioautographic density in the tubulin region of the gel was apparent in proteins translated

* To whom correspondence should be addressed.

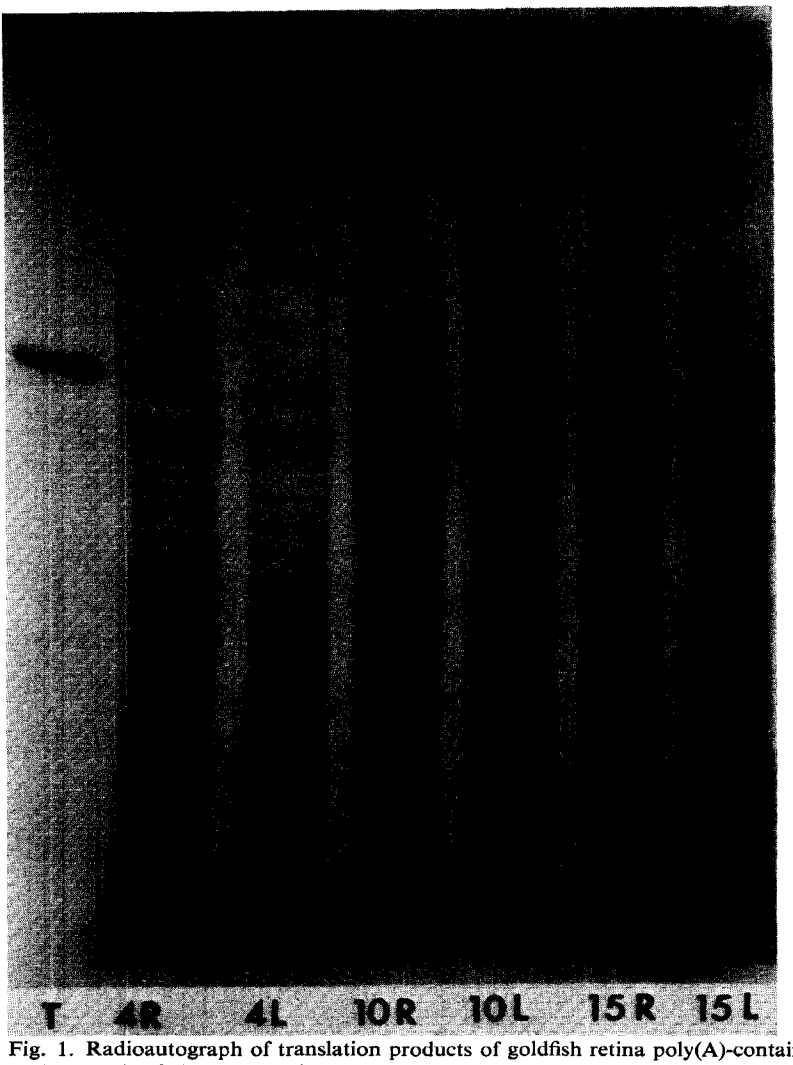

Fig. 1. Radioautograph of translation products of goldfish retina poly(A)-containing RNA. Intraorbital crush of the right optic nerve in goldfish (*Carassius auratus*, 6–7 cm in body length) and removal of retinas was performed as previously described[1]. The left optic nerve remained intact so that the left retina served as a control. Retinal RNA was prepared from the post-mitochondrial supernatant by a SDS–phenol–chloroform procedure, in the presence of Macaloid to inhibit any contaminating RNAse[1]. All solutions and glassware were sterilized. Poly(A)-containing RNA was isolated by affinity chromatography on oligo(dT)-cellulose (Collaborative Research, Type T-3)[1] with the inclusion of 1 mM EDTA in the chromatographic buffers. Carrier yeast transfer RNA (Sigma Chemical) was added to pooled poly(A)-containing RNA fractions to a final concentration of 0.75 A_{260} unit/ml. Ethanol-precipitated RNA was washed once in cold 95% ethanol, dried under nitrogen, redissolved in 50–100 μl of sterile 5 mM K^+–HEPES buffer, pH 7.6, containing 0.1 mM $MgCl_2$ and stored at —70 °C. Approximately 25 μg of cytoplasmic poly(A)-containing RNA could be obtained from 60–75 goldfish retinas. Translation of RNA in a preincubated 'S30' wheat germ extract was carried out essentially as described by Roberts and Paterson[13]. The incubation mixture (100 μl) contained: 20 μl of S30 lysate, 20 mM K^+–HEPES buffer, pH 7.6, 2 mM dithiothreitol, 1 mM ATP, 220 μM GTP, 8 mM creatine phosphate, 47 μg/ml of creatine phosphokinase, 75 mM KCl, 3.5 mM $MgCl_2$, 95 μM spermidine, and 19 amino acids (without methionine), 20 μM each. Ten to 20 μCi of [^{35}S]methionine (New England Nuclear, translation grade, 500–600 Ci/mmol) were added to each mixture. Poly(A)-containing RNA (5–10 μg) was then added and the mixture incubated at 25 °C for 60 min. The reaction was terminated by chilling on ice. Proteins were isolated by centrifugation at 100,000 × g for 1 h, followed by precipitation with 1–2 ml of acetone, centrifugation, and washing with 5 ml of acetone. The final protein pellet was redissolved in 50–100 μl of 62.5 mM Tris·HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol and 5% mercaptoethanol and was stored at —70 °C. Slab gel electrophoresis was performed as described by O'Farrell[12] with minor modifications. Gel slabs were stained and dried under vacuum prior to exposure to Kodak Royal X-Omat film. Abbreviations: T, [^{35}S]methionine labeled goldfish retina tubulin prepared by sequential assembly-disassembly of microtubules[14]; 4R, translation of poly(A)-containing RNA from 4 day post-crush retinas; 4L, 4 day controls; 10R, 10 day post-crush retinas; 10L, 10 day controls; 15R, 15 day post-crush retinas; and 15L, 15 day controls.

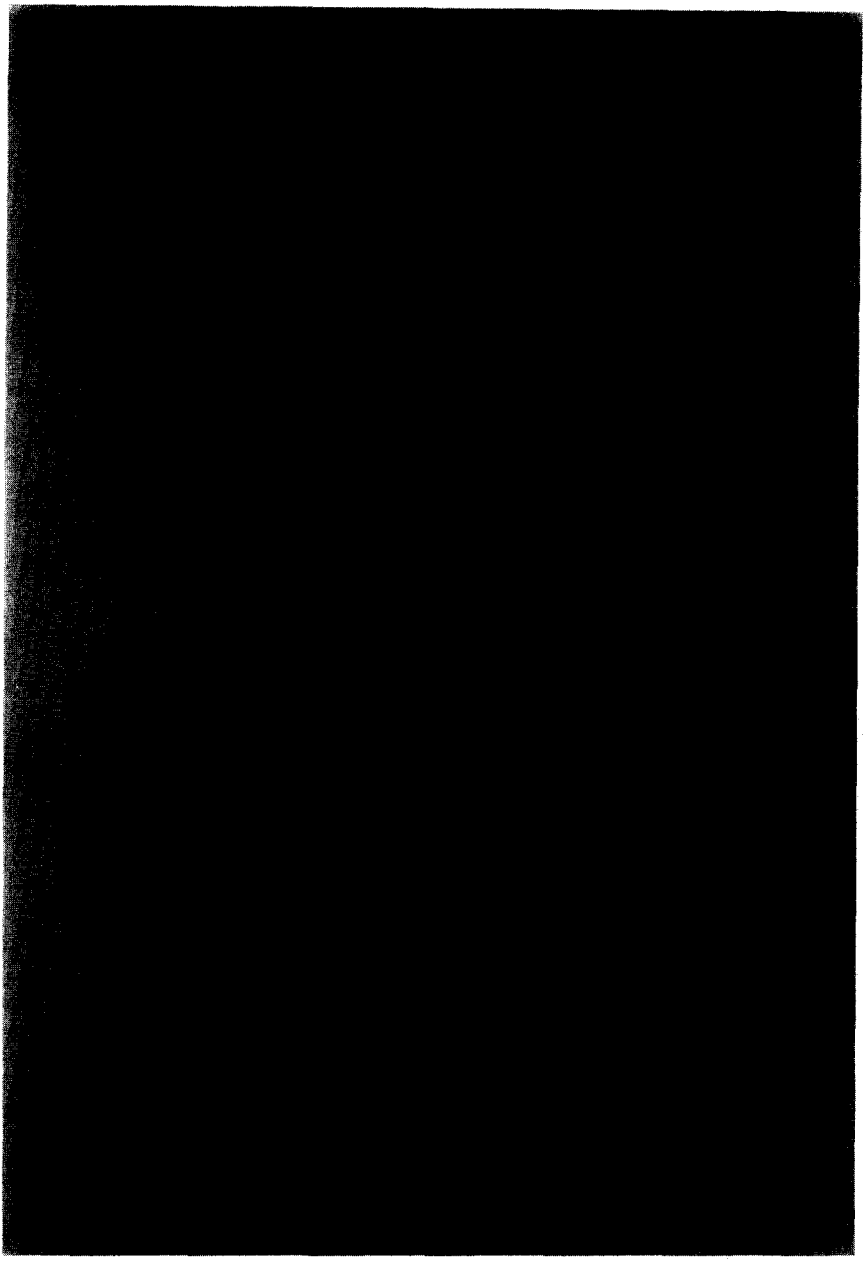

Fig. 2. Radioautograph of a region of a gel following two-dimensional electrophoresis[12] of the translation products of poly(A)-containing RNA from 10 day post-crush retinas. Experimental protocol was as described in the legend to Fig. 1. [^{3}H]Methionine-labeled goldfish retina tubulin was added to the sample prior to electrophoresis. Following radioautography, the tubulin area was cut out and radioactivity in ^{35}S and ^{3}H was determined.

from post-crush compared with control retinal RNA. Densitometric scanning of the latter indicated that tubulin represented 1.37–1.70% of the total labeled protein in the controls and was increased 25–200% in the post crush samples ($P < 0.05$). Two-dimensional gel electrophoresis of cell-free synthesized [^{35}S]labeled proteins (Fig. 2), together with a purified goldfish [^{3}H]tubulin marker, confirmed the identity of [^{35}S]tubulin and suggested that both the alpha and beta tubulin subunits were equally labeled. The two labeled tubulin regions identified by radioautography also corresponded well with those seen from the Coomassie blue-stained carrier brain tubulin.

The observed presence of increased retinal tubulin mRNA 4 days following nerve crush compares well with that anticipated from the time course of enhanced tubulin labeling in the goldfish retina following optic nerve crush[6]. In contrast, increases in cytosolic poly(A)-containing RNA labeling in the retina become significant only 10 days following crush and are maximal at 13 days[1]. These results indicate that a selective cytoplasmic increase in tubulin mRNA may precede a general enhancement of mRNAs following nerve injury. They do not indicate the basis of the mRNA increase, e.g. altered mRNA turnover, processing, etc.

The present findings should be viewed in the context of a vast histological literature describing the effects of axotomy on the neuronal nucleus. These data have suggested that RNA and protein synthesis are augmented in the perikarya of injured neurons that have the ability to regenerate their cut axons. Currently available tools of cell biology permit us to begin to characterize these macromolecules. The results clearly indicate that an increase in retinal tubulin labeling seen following optic nerve axotomy is due, at least in part, to increased availability of its messenger RNA. These results raise the question of whether intrinsic neurons of higher vertebrates also respond to axotomy by increased transcription and translation of structural protein. The answer would bear directly on whether the failure of some intrinsic neurons to regenerate represents an inherent property of the cell body or a block at the axotomy site, such as that arising from glial infiltration.

This work was supported by a grant from the National Institutes of Health (NINCDS 015287). H.R.B. and R.D.W. were postdoctoral scholars supported respectively by MH013831 and GM06138.

1 Burrell, H., Dokas, L. and Agranoff, B. W., RNA metabolism in the goldfish retina during optic nerve regeneration, *J. Neurochem.*, 31 (1978) 289–298.
2 Cragg, B. G., What is the signal for chromatolysis? *Brain Research*, 23 (1970) 1–21.
3 Grafstein, B., The nerve cell body response to axotomy, *Exp. Neurol.*, 48 (1975) 32–51.
4 Guth, L., History of central nervous system regeneration research, *Exp. Neurol.*, 48 (1975) 3–15.
5 Gunning, P. W., Kaye, P. L. and Austin, L., In vivo RNA synthesis within the rat nodose ganglia, *J. Neurochem.*, 28 (1977) 1237–1240.
6 Heacock, A. M. 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.
7 Jacobson, M. and Gaze, R. M., Selection of appropriate tectal connections by regenerating optic nerve fibers in adult goldfish, *Exp. Neurol.*, 13 (1965) 418–430.
8 Kerr, F. W. L., Structural and functional evidence of plasticity in the central nervous system, *Exp. Neurol.*, 48 (1975) 16–31.

9 Lieberman, A. R., Some factors affecting retrograde neuronal responses to axonal lesions. In R. Bellairs and E. G. Grey (Eds.), *Essays on the Nervous System*, Clarendon Press, Oxford, 1974, pp. 71–105.
10 Lieberman, A. R., The axon reaction: a review of the principal feature of the perikaryal responses to axon injury, *Int. Rev. Neurobiol.*, 14 (1971) 49–124.
11 Murray, M. and Grafstein, B., Change in the morphology and amino acid incorporation of regenerating goldfish optic neurons, *Exp. Neurol.*, 23 (1969) 544–560.
12 O'Farrell, P. H., High resolution two-dimensional electrophoresis of proteins, *J. biol. Chem.*, 250 (1975) 4007–4021.
13 Roberts, B. E. and Paterson, B. M., Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ, *Proc. nat. Acad. Sci. (Wash.)*, 70 (1973) 2330–2334.
14 Shelanski, M. L., Gaskin, F. and Cantor, C. R., Microtubule assembly in the absence of added nucleotides, *Proc. nat. Acad. Sci. (Wash.)*, 70 (1973) 765–768.
15 Watson, W. E., An autoradiographic study of the incorporation of nucleic acid precursors by neurones and glia during nerve regeneration, *J. Physiol. (Lond.)*, 180 (1965) 741–753.