

EVIDENCE FOR THE GLIA-NEURON PROTEIN TRANSFER HYPOTHESIS FROM INTRACELLULAR PERFUSION STUDIES OF SQUID GIANT AXONS

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ABSTRACT

Incubation of intracellularly perfused squid giant axons in [³H]leucine demonstrated that newly synthesized proteins appeared in the perfusate after a 45-min lag period. The transfer of labeled proteins was shown to occur steadily over 8 h of incubation, in the presence of an intact axonal plasma membrane as evidenced by the ability of the perfused axon to conduct propagated action potentials over this time-period. Intracellularly perfused RNase did not affect this transfer, whereas extracellularly applied puromycin, which blocked *de novo* protein synthesis in the glial sheath, prevented the appearance of labeled proteins in the perfusate. The uptake of exogenous ¹⁴C-labeled bovine serum albumin (BSA) into the axon had entirely different kinetics than the endogenous glial labeled protein transfer process. The data provide support for the glia-neuron protein transfer hypothesis (Lasek, R. J., H. Gainer, and J. L. Barker. 1976. *J. Cell Biol.* **74**:501-523; and Lasek, R. J., H. Gainer, and R. J. Przybylski. 1974. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1188-1192).

The technique of intracellular perfusion of the squid giant axon whereby artificial internal perfusion fluids could be used to replace axoplasm with no detrimental effects on excitability of the axon (1, 10) represented a major advance in neurophysiology (for reviews of the perfusion technique and its impact on electrophysiology, see references 4 and 8). Very little use of this experimental system has been made by biochemists, despite the fact that it represents a unique system to probe by covalent labeling techniques (2) the proteins of the inner surface of an electrogenic membrane (3).

In this paper the intracellular perfusion technique is used as an independent test of the glia-neuron protein transfer hypothesis described in

the preceding report (6). If this hypothesis is correct, then: (a) the perfusate of perfused giant axons should contain significant TCA-precipitable radioactivity, i.e., newly synthesized proteins, when the giant fiber is incubated in [³H]leucine; (b) the appearance of labeled protein in the perfusate should follow a lag period if the protein is synthesized in the adaxonal glia and subsequently transferred into the axon; and (c) the inclusion of high concentrations of RNase in the internal perfusion fluid should not significantly influence the synthesis of proteins in the glia nor their transfer into the axon. These predictions have been analyzed with the perfusion paradigm. In addition, the kinetics of the transfer of newly synthesized

glial proteins into the perfusate were compared to the kinetics of uptake of a labeled exogenous protein, i.e., bovine serum albumin (BSA), from the external medium.

MATERIALS AND METHODS

Isolated giant axons of the squid (*Loligo pealei*) were partially cleaned of small nerve fibers and perfused intracellularly with internal perfusion fluid (composition: 400 mM potassium phosphate buffer at pH 7.3, 4% glycerol [vol/vol], and 5 mM ATP as the potassium salt) by the method described elsewhere (8). In some experiments, RNase at a concentration of 1 mg/ml was added to the internal perfusion fluid. The perfusion technique used consisted of the double cannula method illustrated in the upper inset in Fig. 2. The smaller, inlet cannula (~100–150 μm OD) and the larger, outlet cannula (~300–350 μm OD) are inserted in opposite ends of the axon and slowly advanced intra-axonally towards one another. The smaller cannula is then placed concentrically within the lumen of the larger cannula. The larger cannula serves to core out the central region of the axoplasm, and internal perfusion is initiated by slowly separating the two cannulae, while simultaneously increasing the perfusion pressure on the inlet cannula by raising the level of the perfusion fluid reservoir (see Fig. 2), and applying slight negative pressure to the end of the outlet cannula. The core of axoplasm is quickly washed out of the outlet cannula by the flowing perfusion fluid, thereafter allowing a relatively constant rate of flow of perfusate. Unlike the procedures often used in electrophysiological experiments with perfused axons, in these experiments no proteolytic enzymes were used to digest the residual axoplasm. Thus, much of the axoplasm remained in the axon at the beginning of the perfusion. During the course of the perfusion, this residual axoplasm is probably eroded away continually. The length of the perfusion zone, i.e., the distance between the tips of the cannulae, was maintained at 10 mm, and the axons ranged in diameter between 400 and 700 μm . The rates of flow of the internal perfusion fluid ranged between 15 and 30 $\mu\text{l}/\text{min}$, and the temperature of the experiments was 18.5–21°C. Throughout the course of the perfusion experiments, action potential amplitudes (~120 mV) and conduction velocities (~24 M/s) were monitored in each axon as a measure of the integrity of the axon membrane. In any case, where there was evidence of conduction block or marked decrease in action potential amplitude, the data from the experiment were discarded.

After the onset of the perfusion, the giant fiber was incubated in 1 ml of Millipore-filtered seawater (Millipore Corp., Bedford, Mass.) containing 100 μCi of L-[4,5- ^3H]leucine (sp act, 50 Ci/mmol, New England Nuclear, Boston, Mass.). The perfusate was collected from the outlet cannula at 5-min intervals either in small tubes or directly on 3 MM filter pads for TCA precipitation, as described elsewhere (6). In some experiments, puromycin (50 $\mu\text{g}/\text{ml}$) was included in the external incubation medium. In several experiments, the labeled perfused

axons were removed from the perfusion chamber and the small fibers were dissected away. The 10-mm length of the perfusion zone was then extruded to remove any residual axoplasm, and the axoplasm was combined with the perfusate for an evaluation of the total "axoplasmic" TCA-precipitable cpm. The remaining 10-mm length of sheath was also analyzed for TCA-precipitable cpm, thereby providing the ratio of labeled proteins appearing in the axoplasm and sheath for comparison with related data from the extrusion studies (6).

The radioactively labeled BSA used in the uptake studies was made by the reductive alkylation method of Rice and Means (9), using [^{14}C]formaldehyde (50 mCi/mmol). The labeled BSA ran as a single peak of radioactivity when analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS). In these studies, the perfused axons were incubated in millipore-filtered seawater containing 300 $\mu\text{g}/\text{ml}$ of labeled bovine serum albumin (sp act, 20,000 cpm/ μg). The radioactivity counting techniques used have been described elsewhere (6).

RESULTS

Incubation in [^3H]Leucine

Data from nine experiments in which perfused axons were incubated from the start of perfusion up to 4 h in [^3H]leucine are illustrated in Fig. 1. Perfusate was collected at 5-min intervals after the introduction of the labeled leucine into the external medium, and each sample was analyzed for TCA-precipitable radioactivity. The data in Fig. 1 demonstrate that: (a) no labeled protein could be detected in the perfusate until about 45–60 min after the start of incubation, and (b) the appearance of labeled protein in the perfusate approached a relatively steady level after about 1.5 h and continued at this level for the remainder of the incubation period. With regard to the latter point, it is apparent from Fig. 1 that there was considerable variation in the amount of labeled protein in the consecutive data points from any individual axon. We do not know the cause of this variation. It may be due to a slow, uneven erosion of the residual axoplasm in the perfusion zone caused by the continual flow of the perfusion fluid. However, whatever the cause of this variation, it demonstrates the limitations of the perfusion technique with regard to certain types of experimental intervention, i.e., in experiments where samples at small time intervals are required.

The above variation can be minimized by longer interval collections of the perfusate as is shown in Fig. 2. In this experiment, perfused axons were incubated in [^3H]leucine from 30 min to 8 h and the TCA-precipitable radioactivity in the perfu-

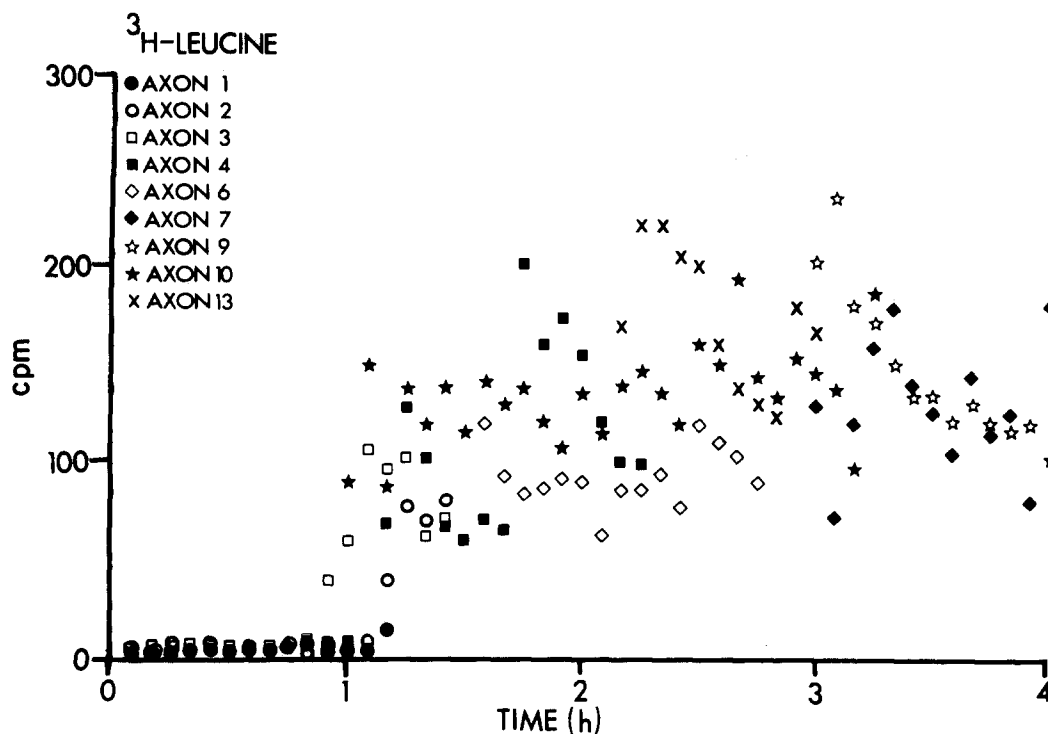


FIGURE 1 Appearance of TCA-precipitable cpm in the perfusate with time after the introduction of [^3H]leucine in the extracellular medium surrounding a perfused squid giant axon (see inset to Fig. 2). Measurement of radioactivity of the perfusate was made at 5-min intervals. Note that labeled proteins did not appear in the perfusate until after 45 min of incubation in the [^3H]leucine, and that there was significant variation thereafter in the absolute cpm per 5-min interval for a given axon (see symbols at upper left for specific axons).

sates was analyzed. The filled circles in Fig. 2 show the results from 14 separate perfused axons under control conditions. As was demonstrated in Fig. 1, the data in Fig. 2 show that there was a 45–60-min lag period in the appearance of labeled proteins in the perfusate, and that the rate of appearance of labeled proteins in the perfusate appeared linear from 2 to 8 h of incubation. Since the axons maintained normal action potentials (120 mV) and conduction velocities over the 8 h of perfusion, we conclude that the transfer of labeled proteins into the axon reported in the preceding paper (6) occurs in the presence of an intact axonal plasma membrane, and therefore was not due to the possible artifactual leakage of labeled proteins from the Schwann cells which might have occurred during the harsher process of extrusion. The radioautographic data presented in the preceding paper (6) also support this conclusion.

The 45-min lag period seen in Figs. 1 and 2 is consistent with the qualitative prediction of such a

lag by the glial-neuronal protein transfer hypothesis (6). While such a lag is seen for the TCA-precipitable cpm in the perfusate, this is not the case for the TCA-soluble cpm. Table I shows the results of such an experiment on a perfused squid giant axon during incubation in [^3H]leucine. During the early periods of incubation, i.e., from 0–30 min, when the perfusate contained virtually no TCA-precipitable radioactivity, the TCA-soluble radioactivity of the perfusate was already near maximal. Thus, considerable [^3H]leucine had entered the intra-axonal space in the early periods of incubation, and yet no newly synthesized proteins appeared in the perfusate. We interpret these data as evidence against the notion of an intra-axonal protein biosynthesis system located on the inner surface of the axonal plasma membrane. Further evidence against this notion is illustrated in Fig. 2. Two axons were perfused with internal perfusion fluid containing 1 mg/ml RNase for 15 min before introduction of the [^3H]leucine into the external medium. During the incubation periods (3 and 5

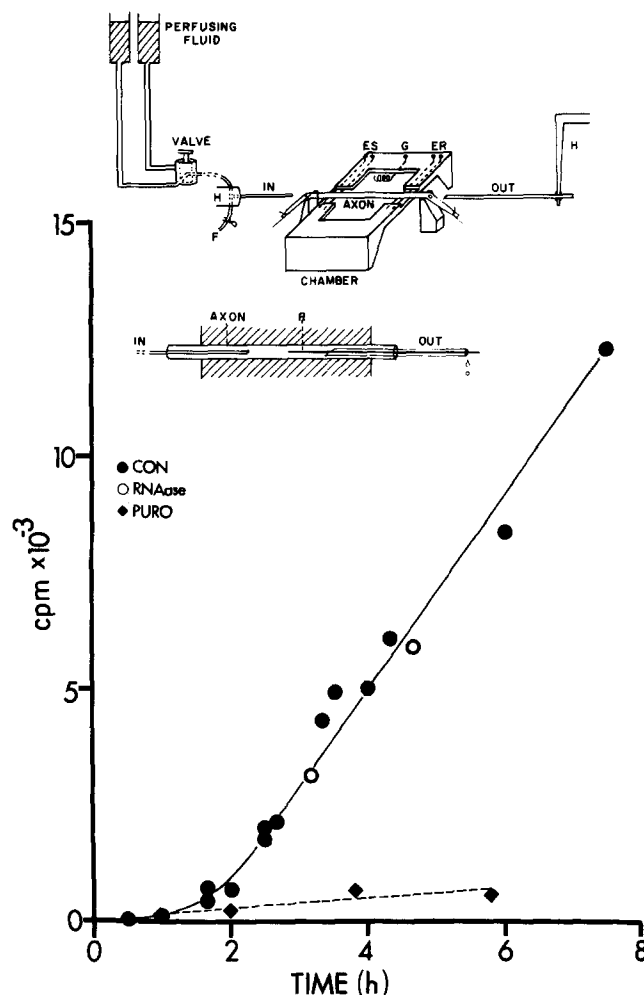


FIGURE 2 Cumulative appearance of TCA-precipitable cpm in the perfusate with time after incubation of the perfused giant axon in [³H]leucine. Closed circles, control axons; open circles, axons perfused with internal perfusion fluid containing RNase (1 mg/ml); filled diamonds, puromycin (50 μg/ml) added to external medium. Each point represents data from a separate perfused axon. Upper insets, diagram of the perfusion technique; *F*, perfusion fluid priming tube; *H*, lucite holders of inlet and outlet pipette; *ES*, external stimulating electrodes; *ER*, external recording electrodes; *G*, ground lead; and *R*, intracellular recording electrode. Inset adapted from Lerman et al. 1969.

h), perfusion with the RNase-containing perfusion was continued. The results of these experiments, depicted in Fig. 2 (unfilled circles), show that there were no effects of intracellularly perfusing RNase on the appearance of labeled proteins in the perfusate or on the electrical properties of the axon. Since the injection of RNase into molluscan neuron cell bodies dramatically reduced protein biosynthesis (discussed in reference 6), we conclude that the postulate of an RNA-dependent protein synthesis system intra-axonally could not account for the labeled proteins in the perfusate.

In contrast, blocking the protein synthesis in the glial sheath by external application of puromycin (Fig. 2, filled diamonds) dramatically reduced the appearance of labeled proteins in the perfusate.

The results presented in Table II indicate that the rates of transfer of newly synthesized proteins were comparable in the perfusion experiments and the experiments employing extrusion in the previous paper (6). After perfusion in [³H]leucine for 2–3 h, seven axons were dissected free of small fibers and analyzed for their ratios of axoplasmic- (transferred) and sheath-labeled proteins. These

TABLE I
Comparison of the Rate of Appearance of TCA-Precipitable and -Soluble cpm in a Perfused Giant Axon Incubated in [³H]leucine.

Sampling time interval*	cpm in Perfusate	
	TCA-soluble	TCA-precipitable
<i>min</i>		
0-15	3,277	0
16-30	3,031	10
31-45	3,486	55
46-60	3,817	118
61-75	4,671	215
76-90	4,510	405
91-105	4,317	393
106-120	3,593	379

* [³H]leucine-containing external medium was placed on the perfused giant axon at zero time, and 15-min perfusate collection intervals were taken for 2 h.

TABLE II
Incorporation of [³H]Leucine in the Axoplasm and Sheath of Perfused Squid Giant Axons

	Axoplasm	Sheath	A/S
	cpm ($\times 10^{-3}$)		%
	3.0	30.7	9.8
	4.6	41.5	11.1
	3.3	20.4	16.2
	3.5	81.0	4.3
	7.9	106.6	7.4
	5.5	52.1	10.6
	9.6	61.6	15.6
Average	5.3	56.3	9.4

* After a 2-3 h perfusion and incubation in [³H]leucine, the nerve fiber was removed from the incubation chamber, and the remaining small nerve fibers were dissected away. The 1-cm length perfusion zone of the axon was extruded to remove any residual axoplasm, and the extruded axoplasm was combined with the perfusate for the analysis of axoplasmic TCA-precipitable cpm. The remaining 1-cm long sheath was similarly analyzed. % A/S = ratio of axoplasm cpm/sheath cpm $\times 100$.

values ranged from 4.3 to 15.6% (average 9.4%, Table II) and were comparable to values determined in extrusion experiments.

Incubation in Exogenous Labeled Protein

In an earlier report, Giuditta et al. (5) found that labeled BSA was taken up by the giant axon of the squid presumably by a pinocytotic mechanism. The kinetics of this uptake were curious, since the labeled bovine serum albumin uptake

occurred during the first 60 min of incubation and declined thereafter (5). We have repeated this experiment and found similar results with the extrusion paradigm. Because a pinocytotic mechanism might be involved in glial-axonal protein transfer (6), we reexamined this issue by the perfusion method.

The results of such experiments with perfused axons incubated in [¹⁴C]-labeled BSA are illustrated in Fig. 3, and appear to confirm the findings of Giuditta et al. (5). There was a burst of uptake of labeled BSA which appeared in the first 60 min of incubation which then declined. Similar results were obtained with the extrusion method (unpublished data). Thus, the kinetics of uptake of this exogenous protein are quite different from the kinetics of uptake of the endogenously synthesized protein of glial origin (see Fig. 1).

DISCUSSION

The above-described studies on the intracellularly perfused squid giant axon lend full support to the glia-neuron protein transfer hypothesis (6, 7). In the presence of a physiologically intact (electrogenic) axonal membrane, newly synthesized glial proteins are transferred into the axon and subsequently appear in the perfusate. The fact that TCA-soluble radioactivity, i.e., the [³H]leucine, appeared in the perfusate well before the TCA-precipitable radioactivity, i.e., labeled proteins, and that intracellular perfusion of RNase did not affect the labeled protein transfer, strongly indicates that the protein biosynthesis mechanism is not intra-axonal.

The qualitative data on the transfer of newly synthesized proteins into the axon in the perfusion studies shown in Fig. 2 agree very well with the data obtained by the extrusion method (see Fig. 2 in reference 6). However, the absolute TCA-precipitable cpm per cm length of axon per hour was 2-3 times higher for the extrusion experiments in comparison to the perfusion experiments. This difference was probably due to the difference in preparation of the axon for incubation in the two types of experiments. In the case of the extrusion paradigm, the axon was dissected completely clean of small nerve fibers, whereas in the perfusion studies a considerable number of small fibers were left around the axon in order to maintain mechanical stability. Thus, there may have been a greater obstacle to the diffusion of the [³H]leucine precursor in the latter. Analyses of autoradiographs of giant fibers surrounded by small fibers

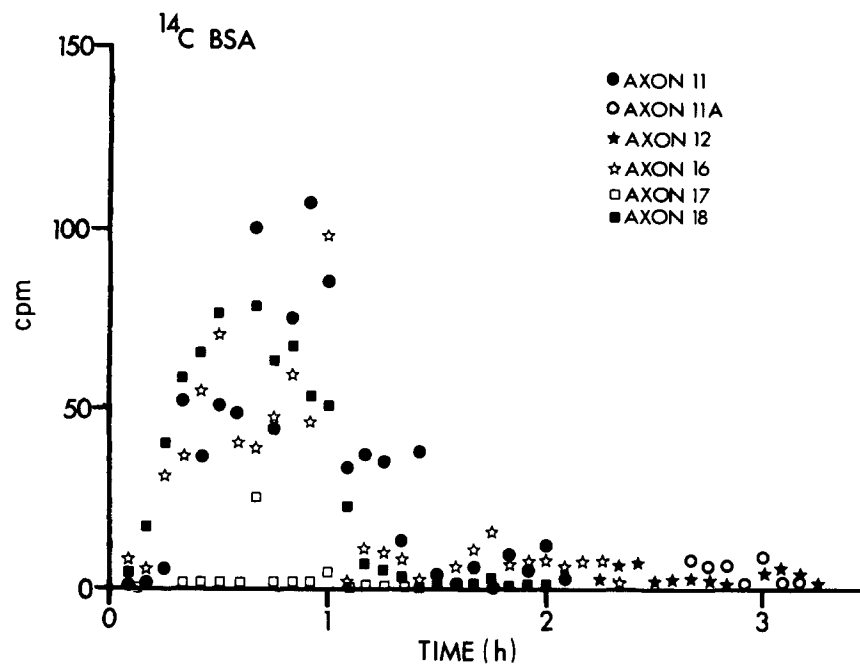


FIGURE 3 Appearance of TCA-precipitable cpm in the perfusate after the introduction of ^{14}C -labeled BSA into the external medium surrounding the perfused giant axon. Note that virtually all the uptake of label occurs in the first 1–1.5 h of incubation. Each symbol represents data from an individual axon.

and those cleaned of small fibers support this interpretation (unpublished results).

The perfusion experiment showed a significant time lag in the appearance of labeled proteins in the perfusate (Fig. 2). The duration of the time lag in the perfusion experiments was ~45–60 min, while in the extrusion experiments a small but significant amount of TCA-precipitable radioactivity was detected in the axoplasm at 15 and 30 min. This apparent difference may result from the differences in the experimental paradigms and may be related to the quantitative differences which were found. In the perfusion experiments, the amount of TCA-precipitable radioactivity per 5-min aliquot ranged between 100 and 200 cpm after 60 min (Fig. 1). In the extrusion experiments, the amount of radioactivity available for analysis was much greater, because the cumulative radioactivity for the whole axon is obtained at each interval. The average values from the extrusion experiments were 258, 963, 2,650, and 10,430 cpm at 15, 30, 45, and 60 min, respectively (6). Therefore, in the extrusion experiment, the relative amount of radioactivity at 15 and 30 min was only 2 and 9% of that at 60 min. If a comparable percentage of radioactivity appears in the axons at 15 and 30 min in the perfusion

experiments, it would amount to <2–9 cpm. This value is too low to be detected reliably and could account for the apparently longer lag period in the perfusion experiments. The qualitative observation of a time lag in both cases is of more importance since it is congruent with the glial-neuronal protein transfer hypothesis, although as was pointed out earlier (6) the transfer kinetics of the giant fiber system were too complex for a definitive analysis.

In the preceding paper (6), exocytosis from the glia coupled to endocytosis in the axon was discussed as a possible mechanism underlying the protein transfer between the cells. In this regard, it is of interest to note that the uptake of exogenous protein, i.e., BSA, possibly by pinocytosis (5), is significantly different from the glia-neuron protein transfer process. The exogenous protein uptake occurs for only the first 60 min of incubation (Fig. 3 and reference 5), whereas the glial-neuron protein transfer process occurs steadily for as long as 8 h (Fig. 2). The reason for the transient nature of the BSA uptake is not clear at present. One possibility is that the presence of BSA in the external medium for 1 h inactivates the pinocytotic mechanism in the giant axon. If this is the case, it is significant that preincubation of the giant

fiber in unlabeled BSA for 1 h did not affect the transfer of labeled glial proteins to axoplasm (see Table 7 in reference 6). Therefore, if the glial-neuronal transfer process is occurring via an exocytotic-endocytotic mechanism, then there must be two independent mechanisms of pinocytosis in the squid giant axon.

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