

# COMPOSITIONAL ANALYSIS OF GROWING AXONS FROM RAT SYMPATHETIC NEURONS

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## ABSTRACT

We describe culture systems for neurons of an adrenergic autonomic ganglion which: (a) permit cultivation of neurons without supporting cells, (b) permit separate harvest of somal and axonal material, and (c) permit direct access to the neuronal surface. The antimetabolites used to suppress supporting cell growth did not have demonstrable effects on neuronal polypeptide synthesis. Rapid neurite outgrowth, which characterized these cultures, was prevented by colchicine or cycloheximide and resumed promptly after their withdrawal. Axons separated from cell bodies showed no incorporation of label from leucine or fucose, but did exhibit incorporation of glucosamine. The major polypeptides present in this neuron, as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, are described. No major differences in polypeptide content were observed when soma and axons were compared. Likewise, there were no differences detected in polypeptides synthesized by neurons in suspension or neurons actively extending processes. Analysis of the polypeptides within the neurites after labeling with amino acids indicated transport at a number of different rates; certain of these polypeptides corresponded in size and transport characteristics to polypeptides observed in the rabbit optic nerve after labeling of retinal ganglion cells. Tubulin and actin have been definitively identified in this cell type (18); we found proteins similar in size and proportionate amounts to be among the rapidly transported soluble polypeptides. The prominent polypeptides observed after several methods of surface labeling are described.

**KEY WORDS** sympathetic neurons · nerve tissue culture · axonal growth · axonal polypeptide transport · neuronal surface membrane

The complex anatomic and metabolic relationships between the various cells of the nervous system complicate study of the activities and composition characteristic of specific cell types. Recent efforts to study the cell biology of neurons and supporting cells include: (a) studies of cells derived from induced or spontaneous tumors

which express certain known neuronal or neuroglial characteristics (for review, see references 27 and 53); (b) studies of "simple" systems in lower forms allowing observations on identified single neurons (e.g., see references 2 and 3); (c) studies of the axonal projections of specific neuronal types, where axons of these neurons may be studied, especially after transport of labeled materials from the neuronal soma (12, 62); and (d) the preparation of specific cell types derived from brain tissue by dissociation and separation proce-

dures, generally utilizing centrifugation techniques (reviewed in reference 52).

An alternate approach is to establish populations of specific cells free of any other cell type in long-term tissue culture. This approach has had limited success, presumably due, at least in part, to the complex "trophic" and other interactions necessary for neuronal survival. The peripheral nervous system, however, contains collections of neurons and supporting cells in ganglia which are easily sustained in tissue culture, and three types of cells may be cultured as pure populations from such ganglia: the adrenergic autonomic ganglion neuron (see review in reference 48), the sensory neuron (45), and the Schwann cell (64). The successful culture of pure populations of normal autonomic (as well as sensory) neurons is dependent upon the availability of nerve growth factor (NGF) and was first accomplished with cultures of dissociated rat superior cervical ganglion cells (7). The culture technique for these neurons was further refined to provide long-term growth, free of non-neuronal cells (42). In the present study, we have employed these earlier culture techniques and, in addition, have modified them by: (a) confining the neuronal somata to discrete explant areas, which allows the separate harvest of neuronal somata and axonal outgrowth, and (b) suppressing supporting cell proliferation. Through the use of this new culture method, differentiated cell morphology is maintained in a manner that allows study of the cell somata and axons separately: in addition, the axonal surface is available for direct study. This paper presents results obtained by using this approach to study the adrenergic autonomic neuron of the superior cervical ganglion of the rat. A previous report has presented evidence that two of the prominent axonal surface proteins closely resemble (and may be identical to)  $\alpha$ - and  $\beta$ -tubulins (16).

## MATERIALS AND METHODS

### *Media for Cell Culture*

Balanced salt solutions and defined media were products of Grand Island Biological Co. (Gibco), Grand Island, N. Y. 1- $\beta$ -D Arabinofuranosylcytosine (ara-C), 5-fluoro-2'-deoxyuridine (FUdR), and uridine were from Sigma Chemical Co., St. Louis, Mo. or Calbiochem, LaJolla, Calif. Radiochemicals were obtained from New England Nuclear Corp., (NEN) Boston, Mass., or Amersham Corp., Arlington Heights, Ill. NGF in the 7S form was prepared from mouse submaxillary glands by the method of Bocchini and Angeletti (5). 1 U

of NGF activity was defined using the bioassay with chick dorsal root ganglion (see reference 60). Human placental serum was obtained from placental blood samples taken at area hospitals. Medium 1: 10 vol % human placental serum, 10 vol % 150 mM KCl, 75 vol % Eagle's Minimal Essential Medium (MEM) with Earle's salt solution, 0.7 vol % 200 mM L-glutamine, 3 vol % 20% glucose,  $10^{-5}$  M ara-C,  $10^{-5}$  M FUdR,  $10^{-5}$  M uridine, 20 U/ml NGF; Medium 2: same as medium 1 but lacking ara-C; Medium 3: 25 vol % human placental serum, 10 vol % 9-day chick embryo extract, 60 vol % Eagle's MEM with Earle's salts, 0.7 vol % 200 mM L-glutamine, 3 vol % 20% glucose, 20 U/ml NGF.

### *Preparation of Superior Cervical Ganglion (SCG) Neuron Cultures*

**EXPLANT TYPE CULTURES:** SCG from perinatal rat pups were removed by sterile technique, taking care to avoid the nearby nodose ganglion of the vagus nerve. Typically, ganglia from two litters of rat pups (20-25 animals) were removed and stored in L-15 medium (Gibco) containing NGF during the dissection. The ganglia were then stripped of their connective tissue sheaths, using fine forceps, and gently teased into three to four pieces. The small pieces, or explants, were placed on collagen-coated Aclar dishes (10). The explants were exposed to medium 1 for the first 5-7 days in vitro and then placed on medium 2 for continued culture. Throughout the period of culture, medium was replaced every 2-3 days.

**DISSOCIATED SCG NEURONS:** Two methods of obtaining this type of culture were used. In both, ganglia stripped of their connective tissue sheaths were incubated at 37°C for 30 min in the presence of 0.25% trypsin (Difco Laboratories, Detroit, Mich.; 1:250) in Hanks' solution lacking calcium and magnesium. After trypsin treatment, the ganglia were gently washed in whole medium three times and triturated through a small-bore Pasteur pipette. The resulting cell suspension was filtered through a 15- $\mu$ m Nitex filter (Tetco Inc., Elmsford, N. Y.). The filtrate of dissociated neurons and non-neurons was then directly plated on a collagen-coated substrate.

Alternately, if preparations of neurons free of non-neurons were desired from the time of initial plating, the filtered cell suspension was further fractionated before plating. The method used, described by McCarthy and Partlow (43), involves the partitioning of neurons and non-neurons based on their affinity for commercial tissue culture plastic surfaces.

### *Incubation with Radioactive Precursors and Autoradiography of Whole Mounted Cultures*

Dishes containing explants, which had been cultured for 3-7 days on medium 2, were rinsed well with *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES)-buffered MEM. In experiments in which ra-

radioactive leucine was to be used, the MEM lacked leucine. When desired, explants were removed by dissection, leaving behind their neuritic fields. Alternately, a cut was made across neuritic fields, thus severing them from their parent somata. In this way, neurites still attached to, or detached from, their supporting cell bodies were subjected to the same incubation conditions.

After rinsing and extirpation or cutting, the cultures were exposed to radioactive precursors in MEM. After the incubation period, cultures were rinsed carefully with MEM containing nonradioactive precursor at 1,000 times the concentration of the radioactively labeled form. The cultures were fixed for 12 h in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2, rinsed three times in buffer without aldehyde, and then rinsed three times in distilled water. After drying, the bottom of the culture dish was cut out and mounted on a microscope slide before coating with Kodak NTB-2 nuclear track emulsion diluted to 50% with distilled water. The emulsion was exposed for 2 wk at 5°C and then developed in Kodak D-19 developer, fixed in Ektaflo, rinsed with distilled water, air dried, and covered with glycerol before observation. Silver grains were counted with the aid of a calibrated ocular reticle and  $\times 40$  phase contrast objective.

#### *Incubation with Radioactive Precursors for Peptide Analysis of Axonal Transport*

Explants that had been maintained in vitro for 2 wk were used. The antimetabolite regimen described above assured removal of nearly all contaminating non-neuronal cells (Fig. 3). Explants were washed free of medium with MEM and then exposed to the desired radioactive precursors in medium 1, lacking FUDR and ara-C and made up with dialyzed serum. When leucine or methionine was used as radioactive precursors, MEM lacking the nonradioactive form of the precursor was used. If the neurites were to be harvested immediately after the labeling period, then the explants were washed three times in phosphate-buffered saline (PBS) containing 100–1,000 times the concentration of nonradioactive precursor. The neurites were then harvested and prepared for analysis. If, after exposure to labeled precursors, the cells were to be maintained in culture, they were washed three times with MEM containing 1,000 times the concentration of unlabeled precursor and then maintained in culture on medium 1, lacking FUDR or ara-C and containing a 1 mM concentration of the nonradioactive precursor.

#### *Cell Surface Labeling Experiments*

Neurites were harvested from explants that were maintained 2 wk in vitro and were free of non-neuronal cells. After washing five times in cold PBS by centrifugation, the neurites were labeled by either the combined glucose oxidase/lactoperoxidase/ $\text{Na}^{125}\text{I}$  method, combined galactose oxidase/ $\text{NaBT}_4$  treatment, or periodate/ $\text{NaBT}_4$  treatment.

**IODINATION OF NEURITES:** Lactoperoxidase (LPO) (EC 1.11.17) from Sigma (no. L-7129) and glucose oxidase (EC 1.1.3.4) from Sigma (no. G-6500) were used to catalyze the iodination of neurites. The enzymes were assayed to determine activities under the conditions used during iodination. The LPO was listed as having 42 purpurogallin U/mg solid. Its activity was found to be 8.7 U/mg, where 1 U of LPO activity is defined as the amount of enzyme necessary to consume 1  $\mu\text{mol H}_2\text{O}_2/\text{min}$ . The glucose oxidase was assayed by coupling with horseradish peroxidase to determine the amount of peroxide generated during reaction with glucose. The glucose oxidase activity was 1,200 U/mg, where 1 U is defined as the amount of enzyme necessary to produce 1  $\mu\text{mol H}_2\text{O}_2/\text{min}$ .

Finally, as a check on the activities of the two enzymes, LPO was titrated against the glucose oxidase by measuring radioiodine incorporation into intact erythrocytes.

The procedure for iodination of the neurites, similar to that described by Hubbard and Cohn (31), was as follows:

Washed neurites were incubated in 500  $\mu\text{l}$  of PBS, pH 7.2, containing 50  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  (NEN, carrier free), 5 mU of glucose oxidase, 5 mU of LPO, and 5 mM glucose. After reaction for 15–30 min at 4°C,  $\text{Na}_2\text{S}_2\text{O}_3$  was added to 10  $\mu\text{M}$  and the neurites were removed from the reaction mixture. The neurites were washed seven times in PBS containing 1 mM KI.

**SODIUM BOROTRITIDE LABELING OF NEURITES:** Sodium borotritide was used to label neurites either alone or after treatment of the neurites with galactose oxidase or periodic acid. The  $\text{NaBT}_4$  was obtained from NEN (185 mCi/mmol). Galactose oxidase (EC 1.1.3.9) from Sigma (No. G-0875) was used after heating at 50°C for 30 min in order to minimize any proteolytic activity that may have been present (57). When assayed under conditions similar to those used in neurite treatment, the enzyme displayed an activity of 27 U/mg, where 1 U of activity was defined as the amount of enzyme necessary to produce 1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2/\text{min}$  at 20°C. The incubation conditions were similar to those given by Steck and Dawson (57). They are given below.

*Galactose oxidase treatment:* Washed intact neurites were incubated in 50 mM  $\text{Na}_2\text{HPO}_4$ , 100 mM NaCl, pH 8.5, containing 1 U of galactose oxidase per ml. After 30 min at 20°C, the neurites were washed and treated with  $\text{NaBT}_4$ .

*Periodate treatment:* Washed, intact neurites were incubated in 100 mM  $\text{Na}_2\text{H}_3\text{O}_2$ , pH 5, containing 1 mM  $\text{NaIO}_4$  at 2°C for 10 min in the dark. After quenching with 50 mM  $\text{Na}_2\text{HPO}_4$ , 100 mM NaCl, 5 mM  $\text{NaAsO}_4$ , pH 8.5, the neurites were resuspended in the phosphate buffer.

*$\text{NaBT}_4$  reduction:* Neurites were suspended in phosphate buffer, and reduced with 1  $\mu\text{Ci}$  of  $\text{NaBT}_4/\text{ml}$  for 1 h. After reduction, the neurites were washed five times in PBS, to remove unbound label.

## Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The discontinuous buffer system of Laemmli (33) was used for SDS-PAGE slab gels of 7.5% or 5–15% linear gradients. Electrophoresis was carried out at 50–100 mV for 8–12 h as indicated by the tracking dye, bromphenol blue. After electrophoresis, the gels were fixed and stained by the method of Fairbanks et al. (17). Gels were photographed on Kodak High Contrast Copy film with a Kodak red filter. Gels were dried onto filter paper for storage and autoradiography on Kodak RPR-54 x-ray film. When tritium-labeled polypeptides were to be detected, fluorography was used as described by Bonner and Laskey (6). Molecular weights of neuritic polypeptides were estimated by comparison with polypeptides of known molecular weight (16). Myosin and spectrin were generous gifts from Dr. M. Willard and Joel Levine.

## Radiolabeling of Polypeptides in Dissociated SCG Neurons

Neurons were purified from trypsin-dissociated SCG by the method of McCarthy and Partlow (reference 42 and above). These cells were then used in experiments designed to test the effects of the antimetabolites on polypeptide synthesis and also to test for differences in polypeptide synthesis between cells that extended axons and those that did not.

To examine the effects of ara-C and FUdR on polypeptide synthesis, dissociated SCG cultures were pretreated for 24 h with the drugs at  $10^{-6}$  to  $10^{-3}$  M in medium 2. At this time, new media was substituted with the addition of [ $^3$ H]leucine (50  $\mu$ Ci/ml) in the presence of the drugs. After a 3-h labeling period, the cells were rinsed with buffer, scraped off the dish, and analyzed by SDS-PAGE combined with fluorography.

To compare leucine incorporation into the polypeptides synthesized by neurons that actively extended processes versus those that did not, neurons prepared as described above were held in suspension or plated on collagen-coated dishes. After 24 h under such culture conditions, the neurons were labeled for 4 h with 50  $\mu$ Ci/ml [ $^3$ H]leucine in medium 2. Some samples additionally had colchicine present during culture and/or the labeling period. After labeling, the cells were harvested by centrifugation or scraping of the culture dish (in the case of plated cells) and analyzed for their radioactive polypeptide content as above.

## RESULTS

### Growth Characteristics of the Three Types of SCG Neuron Cultures

**EXPLANTS:** When pieces of SCG are placed on a collagen-coated substrate, initiation of neurite growth takes place within 24 h and the

expansion rate of neuritic fields is high. However, no growth or only very scanty growth takes place when explants are cultured on poly-lysine, poly-ornithine, or agar substrates. This is in contrast to the behavior of dissociated neurons, many of which attached and extended neurites on these substrates or even on uncoated tissue culture plastic. This property of neurite growth from explants limited the culturing methods to those that utilized collagen-coated substrates when neurite harvests are desired.

Neurite growth rates of these explants are shown in Fig. 1. Although these observations were made during the first few days in culture, there is reason to believe that growth continues at a rapid rate. For example, many explants have

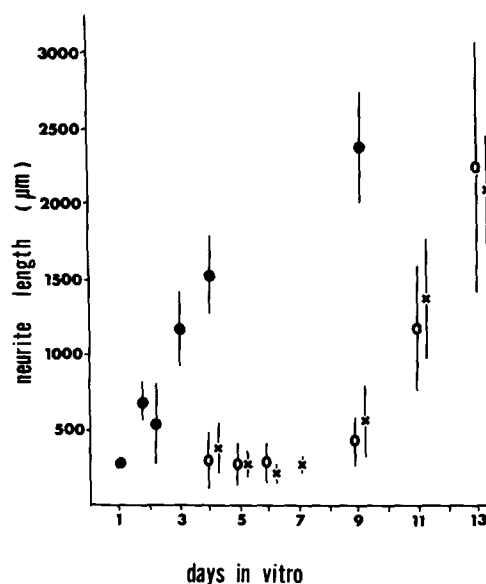
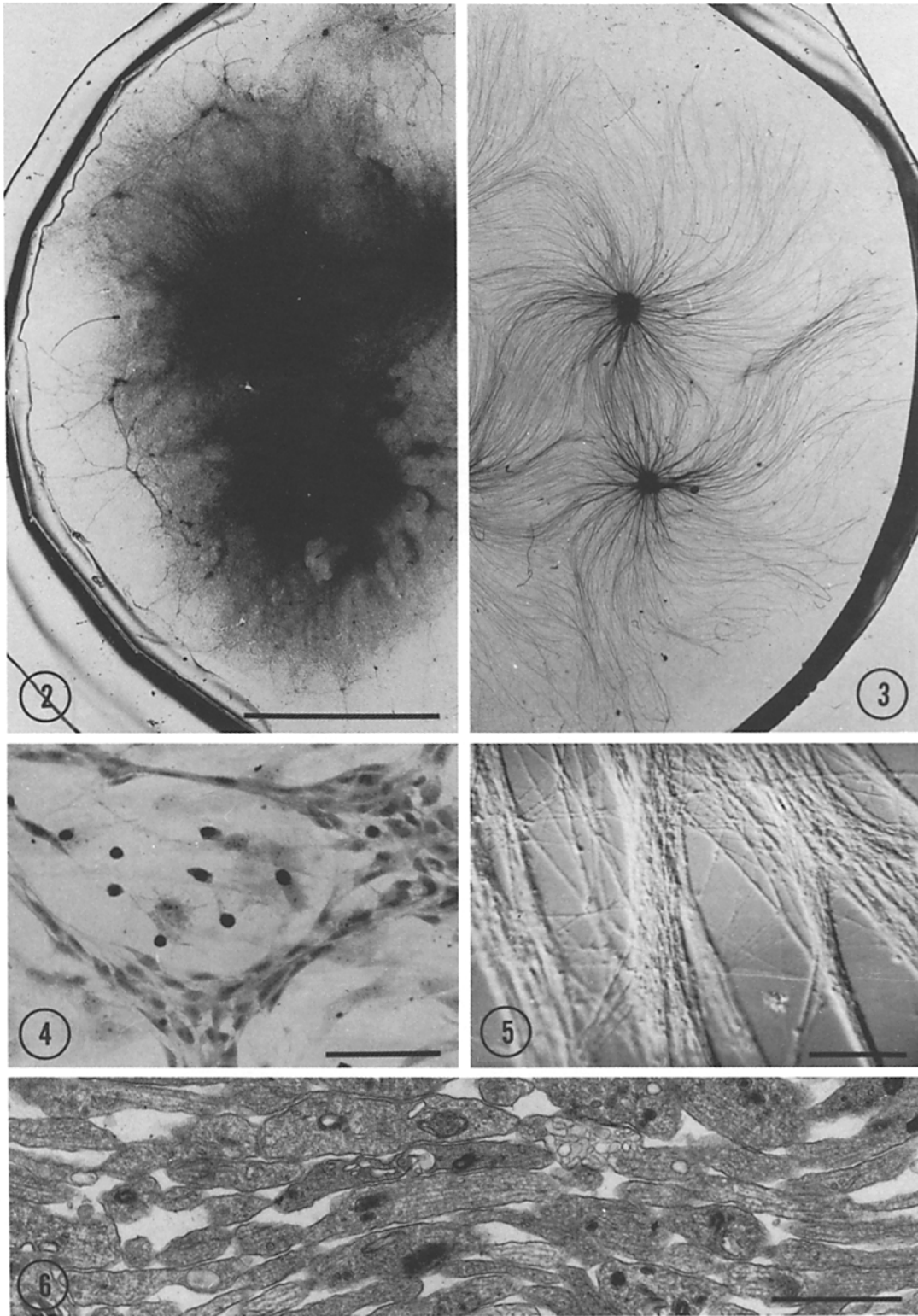


FIGURE 1 Growth rate of SCG neurites. Explant cultures of the type shown in Fig. 3 were set up in medium 2, with refeeding three times each week. Neurite lengths were measured on living cultures, the length being computed from the average of six measurements about each explant. Data on normal neurite outgrowth were gathered using over 120 explants set up at three different times, with each explant being measured on multiple occasions. ●, Explants cultured in medium 2; ○, explants cultured in medium 2 with 5  $\mu$ g/ml cycloheximide present from days 2–8 in vitro; x, explants cultured in medium 2 with 50 nM colchicine present from days 2–8. Data presented in Table I and other experiments not shown indicated that cycloheximide at 5 mg/ml inhibited leucine incorporation into TCA or formaldehyde precipitable macromolecules by 95% or more. Bars represent  $\pm 2$  SD.



been obtained with neurites in excess of 5 mm after 2 wk growth. These figures are probably maximum growth rates under the conditions described, for it is not possible to determine the proportion of neurites that are slower growing and hidden in the outgrowth. From such observations, the neurites appear to be elongating at a rate of over 300  $\mu\text{m}/\text{day}$  during the early culture period.

Fiber outgrowth as depicted in Figs. 2-5 was dependent upon the presence of NGF in the culture medium, and dissociated neurons did not survive in its absence (references 13 and 40, among others). Neurons exposed to cycloheximide or colchicine, even in the presence of NGF, ceased fiber outgrowth and did not resume growth until the agents were removed (data in Fig. 1). After removal of the drugs (at 8 days), fiber outgrowth resumed at approximately the same rate as was seen in the initial outgrowth in untreated explants. The growth rate of neurites treated with the antimetabolites as described was similar to that shown by the control cultures in Fig. 1. By 14 days in vitro, the neuritic mass represented 30-50% of the total cellular mass (explants plus neurites) on the basis of protein content.

The histological effects of combined antimetabolite treatment can be seen in Figs. 3 and 5 as compared to Figs. 2 and 4. Although a number of methods of reducing non-neuronal cell proliferation were tried, combined ara-C and FUdR gave

the most consistent results. In untreated cultures, the outgrowth zone was filled with cells derived from connective tissue and Schwann cells contained within the explant.

**DISSOCIATED WHOLE SCG:** When a filtered, trypsin-dissociate of the SCG is plated, at least three cell types are found. After 12 h in vitro, these cultures consist of 50-75% neurons, the remaining cells being fibroblasts or Schwann cells. The yield of neurons by this method was about 50-70% (up to 15,000 of the 24,000 neurons in the newborn rat SCG; C. D. Ross, manuscript in preparation; reference 30). If plated in medium 3, a permissive medium for non-neuronal cell growth, fibroblasts rapidly formed a monolayer upon which a neuronal network, with its accompanying Schwann cells, grew (Figs. 7 and 9). After 7-10 days in vitro on medium 2, however, these cultures were essentially free of non-neuronal cells (Figs. 8 and 10). After this treatment, switching to a medium permissive for fibroblast growth did not result in the proliferation of non-neuronal cells. Thus, monolayer networks of SCG neurons may be generated in high yield by this simple culture technique.

**PRIMARY CULTURES OF PURIFIED NEURONS:** Although the method described above resulted in purely neuronal-dissociated cell cultures after 7-10 days growth, selective preplating of trypsin-dissociated ganglia made possible the generation of predominantly neuronal cultures in

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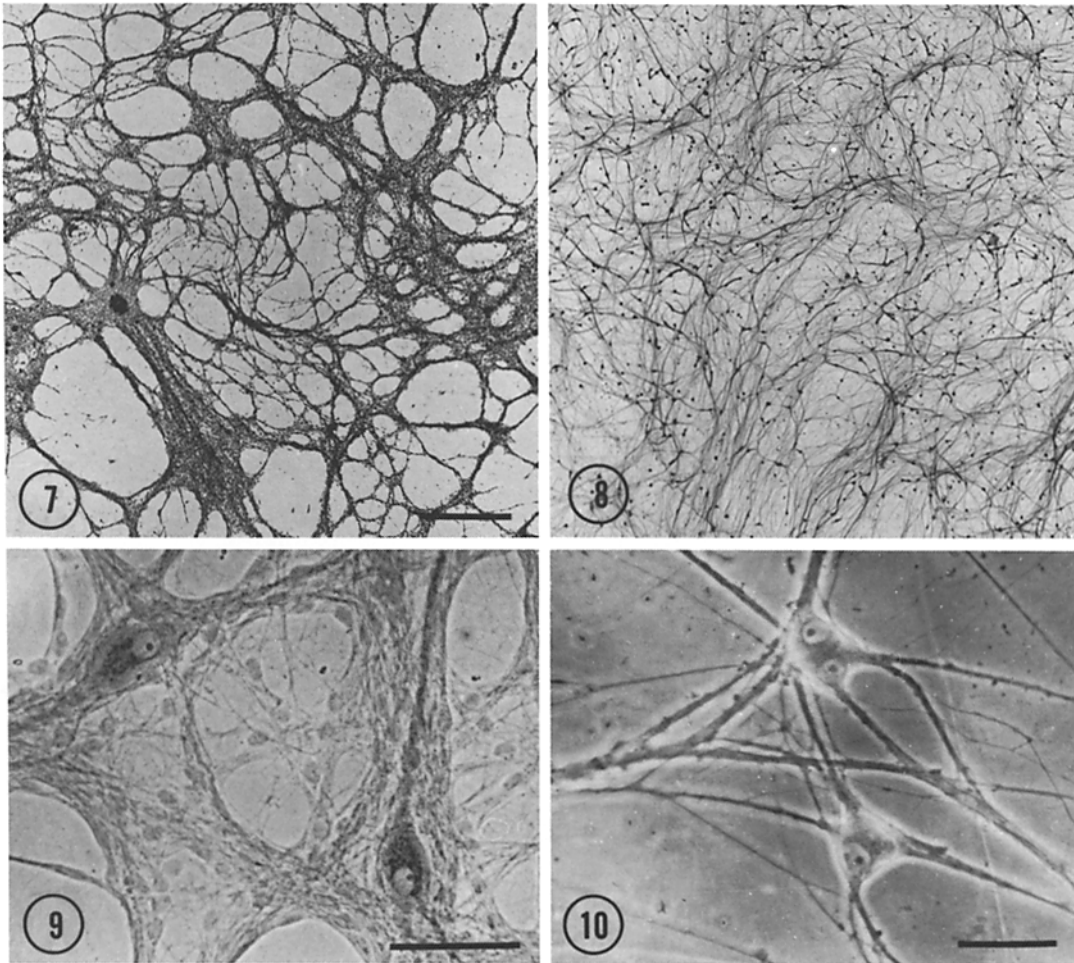
**FIGURE 2** SCG explants cultured for 15 days. Explants were set up on medium 3 and refed every 2 days. Original ganglion explant areas are now obscured by the dense outgrowth of non-neuronal cells. At the edge of the dish, individual bundles of neurites accompanied by Schwann cells can be seen on a background of fibroblasts. Toluidine blue stain. Bar, 5 mm.  $\times 6$ .

**FIGURE 3** SCG explants cultured for 20 days. Explants were set up on medium 1 for the first 7 days in vitro, and then maintained on medium 2 with refeeding every other day. By this regimen, the non-neuronal cells were eliminated and the neurons were allowed to develop in a manner that allowed the separation of somata and growing axons. Sudan black stain after  $\text{OsO}_4$  fixation.  $\times 6$ .

**FIGURE 4** Higher-power photograph of neurite bundles from Fig. 2, with accompanying Schwann cells. The neurites are completely obscured by the ensheathing Schwann cells and rest upon a bed of nearly confluent fibroblasts. Toluidine blue stain. Bar, 50  $\mu\text{m}$ .  $\times 350$ .

**FIGURE 5** Nomarski interference optics photomicrograph of neurite bundles. This photograph was taken of a living culture that had been treated like the explants in Fig. 3. The bundles are composed of dozens of individual neurites, a few of which can be seen bridging the bundles. Bar, 20  $\mu\text{m}$ .  $\times 750$ .

**FIGURE 6** Electron micrograph of isolated neurites. Neurites were dissected from a culture such as that pictured in Fig. 3, and pelleted by centrifugation in phosphate-buffered saline at 5,000 g. The pellets were fixed in glutaraldehyde followed by  $\text{OsO}_4$ , and embedded in Epon/Araldite for thin sectioning. Neurites are long, thin structures  $\sim 0.1$ - $0.2 \mu\text{m}$  in diameter. They contain an abundance of microtubules, smooth endoplasmic reticulum, and mitochondria. Bar, 1  $\mu\text{m}$ .  $\times 20,000$ .



**FIGURE 7** Dissociated SCG. After culture of a trypsin-dissociated ganglion in medium 3, all cell types contained in the ganglion are found. Single neurons and small groups of neurons are connected by neuritic bundles upon which Schwann cells proliferate. Fibroblasts are also present in such a culture system. Toluidine blue stain. Bar, 1 mm.  $\times 12$ .

**FIGURE 8** Dissociated SCG neurons, 6 wk in vitro. When dissociated SCG are cultured for 7-10 days on medium 2, the non-neuronal cell population is eliminated. Such cultures can then be maintained free of non-neuronal cells for at least 8 wk, even with medium 3 which is permissive for non-neuronal cell growth. Sudan black stain after  $\text{OsO}_4$  fixation.  $\times 12$ .

**FIGURE 9** This is a higher-magnification picture showing two neurons and many non-neuronal cells, such as would be found in the type of culture pictured in Fig. 7. The Schwann cells assume the typical satellite cell configuration upon the somata and ensheath but do not myelinate the neurites. Sudan black stain after  $\text{OsO}_4$  fixation. Bar, 50  $\mu\text{m}$ .  $\times 350$ .

**FIGURE 10** Phase contrast photomicrograph of dissociated SCG neurons free of non-neuronal cells. This picture, of a living culture, demonstrates the appearance of neurons such as those in the fixed and stained culture pictured in Fig. 8. Bar, 50  $\mu\text{m}$ .  $\times 250$ .

<1 day from the time of dissection (43). This method served to eliminate non-neuronal cells on the basis of their greater affinity for plastic sub-

strates: with intermittent shaking, neurons that were attached only loosely were resuspended whereas non-neurons were more likely to remain

attached. With 20–30 such cycles during a 4–6 h period, preplating by this technique resulted in enrichment of ganglion dissociates from  $\approx 60\%$  neurons to  $>95\%$  neurons. This enabled one to have neurons free of supporting cells at the time of initial neurite outgrowth, in quantities sufficient for some biochemical studies. These cultures contained single neurons and small clusters of neurons. The yield of cells obtained by this method was less than that following the direct plating of trypsin dissociates due to neuronal cell loss during the preplating steps.

#### Effects of FudR and Ara-C on Leucine Incorporation into Dissociated SCG Neurons

Cultures free of non-neuronal cells exposed to varying concentrations of antimetabolites show no overt morphological signs of toxicity. When polypeptides synthesized by these cells after 3 h of [ $^3\text{H}$ ]leucine labeling in the presence of the antimetabolites were analyzed by SDS-PAGE, with quantitative analysis by fluorography, no consistent changes were present in any of the six molecular weight classes examined (Fig. 11). Although

some variation was observed in individual molecular weight classes from culture to culture, no consistent changes were present that could be ascribed to the use of these antimetabolites.

#### Autoradiographic Experiments with Radioactive Precursors to Macromolecules

To examine the topography of radioactive precursor utilization in SCG neurons, autoradiographic examination of whole cells and isolated neurites was made after incubation with amino acid and sugar precursors. The method of analysis of precursor utilization was checked for reliability using tritiated leucine, and the results for this and the other precursors are given in Table I. Briefly, when the experiments were carried out as previously described, the appearance of leucine-containing macromolecules in the neurites was found to be dependent on time, temperature, the presence of an intact synthetic system (i.e., cycloheximide sensitive), and highly dependent upon connection with the cell body. The fixation and rinsing procedures were capable of reducing background to negligible levels. This system allowed

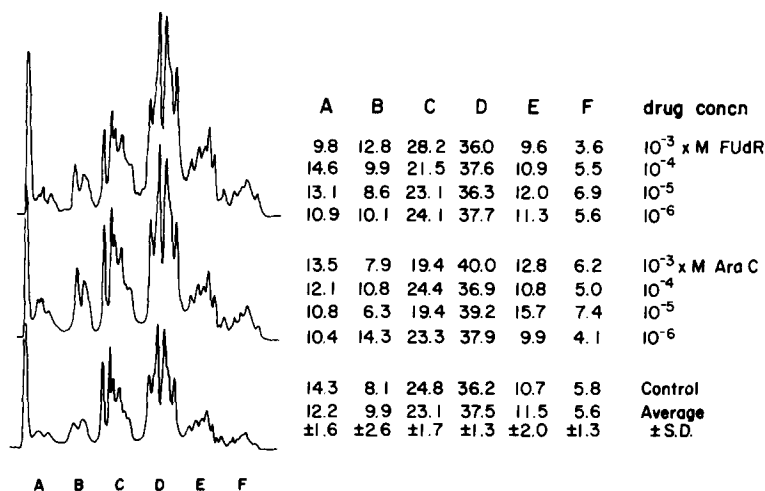


FIGURE 11 Effects of FUDR and ara-C on polypeptide synthesis in dissociated SCG neurons. Cultures prepared by the method of McCarthy and Partlow (42) were incubated with  $50 \mu\text{Ci/ml}$  tritiated leucine for 3 h in order to label polypeptides synthesized during that period. Drug-treated cultures were preincubated with the drug for 24 h before the labeling period (also in the presence of the drug). After labeling, the cultures were rinsed with buffer and prepared for SDS-PAGE. Scans on the left represent densitometric data obtained from fluorographs of gels incubated in the presence of  $10^{-3}$  M FUDR (upper scan),  $10^{-3}$  M ara-C (middle scan), or control medium (lower scan). Data on the right represent the percent contribution of radioactive polypeptides in various molecular weight classes to the total radioactive protein synthesized by the cells. Values given below the control values represent the mean relative contributions for the various polypeptide weight classes in the experimental cultures. The molecular weight assignments of the various weight classes are: group A  $>270$  kilodaltons; group B, 270–190; group C, 190–85; group D, 85–40; group E, 40–30; and group F,  $<30$ .



TABLE I

	Background	Detached neurites	Attached neurites	Fibroblasts
No labeled precursor added	0.06 ± 0.04 (7)	not done	0.15 ± 0.13 (10)	0.17 ± 0.41 (6)
[ <sup>3</sup> H]leucine, 5 μCi/ml				
5°C, 2 h	0.11 ± 0.07 (26)	0.46 ± 0.33 (43)	0.66 ± 0.47 (24)	14.9 ± 1.8 (21)
24°C, 2 h	0.56 ± 0.24 (28)	1.8 ± 1.1 (26)	3.9 ± 1.9 (37)	*
37°C, 2 h	0.50 ± 0.42 (33)	1.3 ± 0.73 (32)	10.7 ± 3.3 (28)	*
37°C, 2 h +				
5 μg/ml cycloheximide	0.12 ± 0.09 (30)	0.60 ± 0.44 (41)	0.66 ± 0.37 (54)	4.7 ± 1.9 (36)
20 μg/ml cycloheximide	0.10 ± 0.08 (25)	0.41 ± 0.38 (24)	0.50 ± 0.34 (45)	3.4 ± 1.3 (31)
[ <sup>3</sup> H]glucosamine, 20 μCi/ml	0.33 ± 0.14 (45)	6.15 ± 1.61 (19)‡	12.5 ± 3.9 (41)	13.1 ± 3.5 (40)
37°C, 2 h				
[ <sup>3</sup> H]fucose, 10 μCi/ml 37°C, 4 h	0.31 ± 0.18 (24)	1.5 ± 1.2 (47)	11.7 ± 3.7 (39)	8.5 ± 2.2 (27)

Precursor utilization by SCG neurites. Explants of SCG cultured from 3–7 days were exposed to radioactive leucine, glucosamine, or fucose to examine autoradiographically the incorporation of these precursors into macromolecules. Immediately before labeling, multiple areas of neurites were detached from their supporting cell somata by cutting across neuritic bundles with a scalpel blade. After labeling, the cultures were washed and fixed in preparation for autoradiography with Kodak NTB-2 emulsion. For each precursor and labeling condition, areas of neurites attached to and detached from neurons were examined along with non-neuronal cells and noncellular areas of the dish. Numbers listed represent grain counts/100 μm<sup>2</sup> of neuritic fields, or grains/100 μm<sup>2</sup> fibroblast cytoplasm, or grains/100 μm<sup>2</sup> substrate (background levels). Data are given as grain counts ± SD; the number of areas examined is indicated in parentheses. Of these precursors, the detached neurites utilized only glucosamine, with that level of incorporation being significantly different from either background or the level reached by neurites still attached to their somata.

\* Grain counts too high to quantitate visually (>50 grains/100 μm<sup>2</sup>).

‡ Significant at  $P < 0.001$

one to directly compare intact and cut neurites along with non-neuronal cells on the same autoradiograph. Thus, even though separate samples were coated with emulsion and processed under differing conditions, direct comparisons were possible.

As shown in Table I, the neurites have little ability to utilize leucine or fucose in the synthesis of larger macromolecules retained after fixation. In the case of glucosamine, cut neurites were able to utilize the precursor. Although a lower level of incorporation was seen with neurites than with the somata present, this level was still well above the background for this experiment and was a consistent finding; it is discussed in some detail below.

#### *Axonal Transport of Polypeptides after Incubation with Radioactive Amino Acids*

When SCG explants were exposed to radioactive leucine, and the neurites and cell bodies examined at later times for the presence of radioactive macromolecules, the results obtained were as presented in Fig. 12. Radioactive proteins appeared in the neurites in a time-dependent fashion. In this experiment, the neurites repre-

sented 40% of the total cell mass on the basis of protein content. By 8–12 h, however, only 15% of the protein made during the pulse period had moved into the neurites. The increase in total neuritic radioactivity was accompanied by an increase in the specific activity of neuritic protein from 100 cpm/μg to 600 cpm/μg, during the chase period. Polypeptides transported at a rate of 1 mm/day or greater would be expected to be present in the neurites by 12 h. The dissection techniques detach neurites within 0.5 mm of the explant; thus a labeled polypeptide must travel that distance before being found in the neurite. Using a longer chase period (data not shown), up to 25% of the protein synthesized during a pulse period was found to be transported over the period of a day. From these experiments, and the data showing that neurites alone are unable to incorporate leucine into macromolecules, it is clear that the cell somata were supplying newly synthesized proteins to their axons in a fashion that may be similar to the well-described phenomenon of axonal transport that occurs in vivo.

It was of interest to determine the transport characteristics of these polypeptides. Five separate transport velocities, represented by unique groups

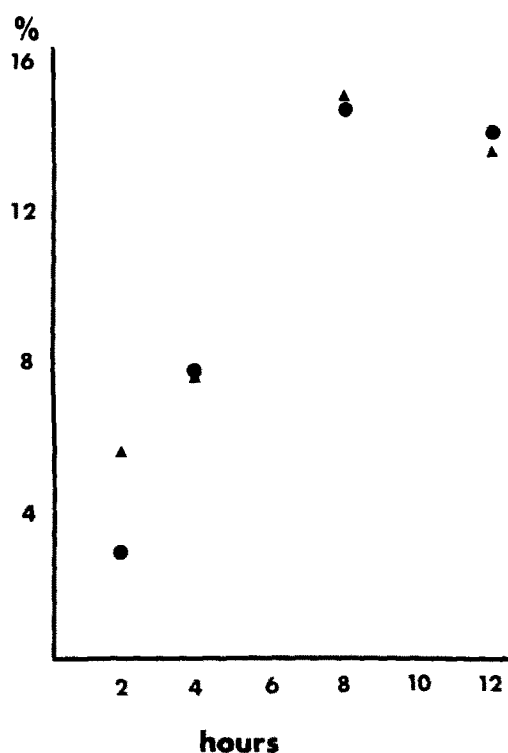


FIGURE 12 Accumulation of acid-precipitable radioactivity in neurites. This figure illustrates two experiments in which the accrual of radioactively labeled macromolecules was demonstrated after a 2-h labeling period with [ $^3\text{H}$ ]leucine. Radioactive leucine ( $50 \mu\text{Ci/ml}$ ) was applied to non-neuronal cell-free explants in medium 2 for 2 h and then removed, and medium containing 1 mM nonradioactive leucine was substituted. At the times indicated, neurites and explants were harvested separately. Each sample was homogenized, and a portion of the homogenate was taken for protein analysis and another was taken for scintillation counting after precipitation onto glass filters with trichloroacetic acid. The ordinate is the percent of the total cell-precipitable radioactivity (somata + neurites) contained in the neurites. The abscissa is the time in hours after initiation of labeling.

of polypeptides, are evident in rabbit retinal ganglion cell neurons (62, 63). In addition, slowly transported polypeptides, including tubulin and possibly neurofilament proteins, have been implicated in axonal growth and regeneration of axons (component SCb in reference 39).

Polypeptides characterizing the SCG neurite as seen after SDS-PAGE are shown in Fig. 13. Neurites, after appropriate pulse and chase times, were analyzed by electrophoresis. As seen in Fig. 14, after 2 h of labeling with tritiated leucine, a

large number of polypeptides were already present in the neurites: these were transported at a rate  $>10\text{--}20 \text{ mm/day}$ . With longer chase periods, the distribution of radioactive polypeptides in the neurites was seen to change. While the contribution of polypeptides in the 60–100 kilodalton range decreased between 2 and 4 h, they exhibited a dramatic increase between 4 and 8 h. This suggested that a number of even more slowly transported polypeptides (velocity 2–5 mm/day) were present in this molecular weight range.

By using [ $^{35}\text{S}$ ]methionine, a shorter pulse period and a more detailed examination of the polypeptides transported was possible. The polypeptides associated with the soluble and particulate fractions from the neurites are illustrated in Fig. 15. In the soluble fraction, polypeptides co-migrating with tubulin and actin (and identified as those two proteins in SCG neurons by peptide maps; reference 18) are the major transported polypeptides, even at the earliest time point (which represents material moving faster than 25–50 mm/day). Time-dependent compositional changes were not seen in this fraction; this observation is apparently different from observations in other systems studied. In the particulate fractions, polypeptides were observed to move at a variety of rates, and time-dependent compositional changes were clearly seen. For instance, band E3 at  $\approx 32$  kilodaltons appeared early in the neurites and was a major fast-transported polypeptide. Band C6 ( $\approx 87,000$ ) and bands B1 and B2 (270,000; 250,000) were present at the first time point and continued to increase throughout the chase period, while additional high molecular weight bands migrating at A1 and A3 appeared at 8 h after the pulse began. In addition, at least one heavily labeled polypeptide band, at 18 kilodaltons, was present at the earliest time point and commenced immediately to become less radioactive, disappearing by 8 h. This polypeptide is particularly interesting because it behaves like band 22 polypeptide of the rabbit retinal ganglion cell neurons. It is transported fast ( $>240 \text{ mm/day}$  in the rabbit; reference 62), 25–50 mm/day in the SCG neurites, and decays rapidly ( $t_{1/2} \approx 3 \text{ h}$  in rabbit).

#### Macromolecules Labeled After Incubation with Carbohydrate Precursors

Fucose and glucosamine were used to metabolically label the glycoproteins of the cell. After incubation with precursor, the polypeptides of the neurites were examined by SDS-PAGE. Fucose

labeled predominantly only one soluble polypeptide of the neurite of ~250 kilodaltons. The particulate fraction, in contrast, contained nine major labeled bands of 270, 250, 140, 100, 92, 58, 53, 47, and 30 kilodaltons (for illustration, see reference 16). In contrast to the paucity of soluble glycoproteins labeled with fucose, gluco-

samine labeling gave rise to >30 labeled soluble polypeptides with major polypeptides of approx. 250, 115, 100, 58, 53, and 47 kilodaltons. With both precursors, the polypeptides associated with the particulate fraction derived from the neurites were most heavily labeled. In the case of glucosamine but not fucose, there was prominent labeling at the gel front in an area where glycolipids would be expected to migrate.

#### Cell Surface Labeling of Neurites

In vitro iodination of SCG neurites resulted in the labeling of ~10 polypeptides with most of the incorporated iodine present in six bands as seen after SDS-PAGE. Fig. 16 demonstrates the prominently labeled polypeptides at 180, 170, 70, 58, 53, and 30 kilodaltons. Less heavily labeled bands are present at 96, 62, and 25 kilodaltons. It should be noted that, since publication of earlier work (16), the high molecular weight bands have been reassigned molecular weights of 180-170 kilodaltons. Less than 2% of the iodine incorporation is present when LPO is omitted from the reaction mixture, even in the presence of the peroxide-generating system, and no radioactive bands are present after SDS-PAGE. Although plasma membrane isolation has not been accomplished to verify the location of the label, >80% of the iodine incorporated is associated with the particulate fraction of these neurites after homogenization and centrifugation of the cell particulates. The band we believe to be actin, a major component of the cytoplasmic polypeptides in these neurites, is not labeled unless the neurites are first homogenized before labeling (data not shown). The polypeptides with apparent mol wt of 180, 170, 58, 53, and 30 kilodaltons can be removed from the intact neurites by brief treat-

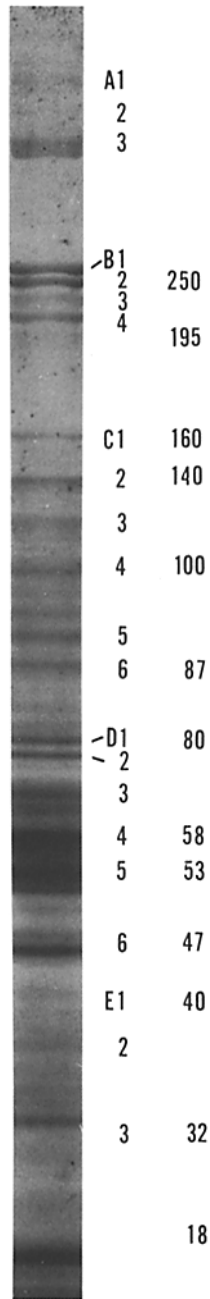


FIGURE 13 Coomassie blue-stained SDS polyacrylamide gel of neuritic polypeptides. Neurites were dissected free from their explants, washed well with buffer, and electrophoresed on 5-15% gradient gel as described in Materials and Methods. Apparent molecular weights were determined by comparison with polypeptides of known size, and are listed on the right, in kilodaltons. We present the tentative nomenclature for reference only. Apparent from such an analysis is the predominance of polypeptides at 58, 53, and 47 kilodaltons. Polypeptides with these molecular weights from the rat SCG neuron have previously been identified as tubulin and actin by Fine and Bray (18).

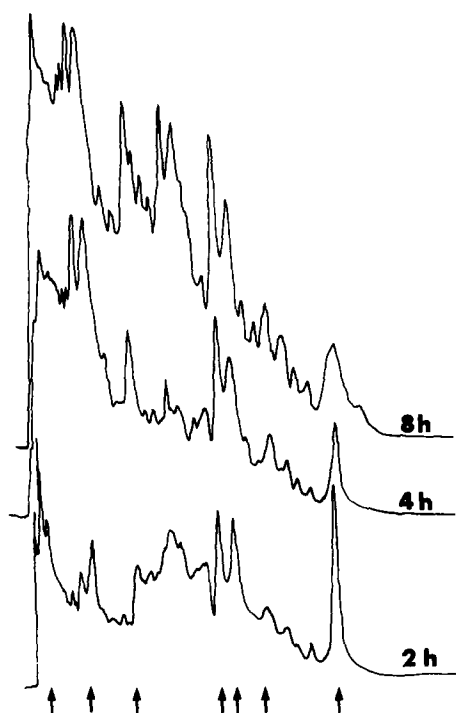


FIGURE 14 Compositional analysis of [ $^3\text{H}$ ]leucine-labeled polypeptides undergoing axonal transport. These results were derived from densitometric scans of fluorographs made from SDS gels. Explant type cultures, free of non-neuronal cells, were exposed to [ $^3\text{H}$ ]leucine (50  $\mu\text{Ci/ml}$ ) for 2 h. At that time and after 2 and 6 more hours of culture in nonradioactive medium, the neurites were dissected free from the explants, and the radioactive polypeptides were separated by SDS-gel electrophoresis. Direction of migration was from left to right, with arrows indicating the apparent mol wt of  $\approx 300$ , 200, 100, 58, 53, 46, and 20 kilodaltons.

ment with trypsin, further supporting the external location of the incorporated label (16).

When neurites were labeled with  $\text{NaBT}_4$  alone or in combination with enzymatic or periodic acid oxidation, labeled polypeptides as shown in Fig. 17 were found. Sodium borotritide alone labeled material that remained at the origin of SDS gels, and also four polypeptides of 230, 220, 145, and 120 kilodaltons. Galactose oxidase treatment specifically catalyzed the labeling of components migrating with apparent mol wt of 160, 93, 90, 58, 53, and 32 kilodaltons. Periodate oxidation resulted in the additional labeling of components with apparent mol wt of  $>300$ , 270, 82, and 45 kilodaltons.

A comparison of the results obtained by meta-

bolic labeling with fucose and external labeling catalyzed by the above methods suggests that polypeptides demonstrated by external labeling may include at least five of the nine major fucose-containing proteins of the neurite (on the basis of their similar mol wt of 270, 58, 53, 47, and 30 kilodaltons).

#### *Polypeptides Synthesized by Neurons in Suspension vs. Neurons that Actively Extend Axons*

Neurons held in suspension after purification from trypsin dissociates formed aggregates, while those which were plated onto a collagen-coated substrate actively extended neuritic processes. By 24 h in vitro, the majority of the plated cells were undergoing axon extension; those plated in the presence of colchicine attached to the substrate but did not extend processes.

After labeling the neurons maintained under these two conditions with [ $^3\text{H}$ ]leucine, the results seen in Fig. 18 were obtained. When band-by-band comparisons were made between experimental groups, no qualitative differences were detected in the polypeptides produced. Neurons in suspension or plated, whether in the presence of colchicine or in its absence, appeared to synthesize similar polypeptides, when analyzed by SDS-PAGE. These results differ from those obtained with neuroblastoma cells, where differences in polypeptide composition have been observed between cells that support processes and those that do not (1, 22, 59).

## DISCUSSION

### *Methodology*

Each of the methods mentioned in the Introduction, designed to delineate specific cellular functions in the nervous system, provides certain advantages and some disadvantages. Cells derived from tumor sources may provide models for studies of certain neuronal attributes, such as opiate receptor function (56) or NGF action (55). These cells may also demonstrate a degree of differentiated function, such as neurotransmitter synthesis and release (24, 54) and neurite extension (25). Many of these cell lines are of unknown source, however, and are unsuitable as models for studies of certain aspects of normal neuronal metabolism. Cells that are separated by density gradient centrifugation or velocity sedimentation,

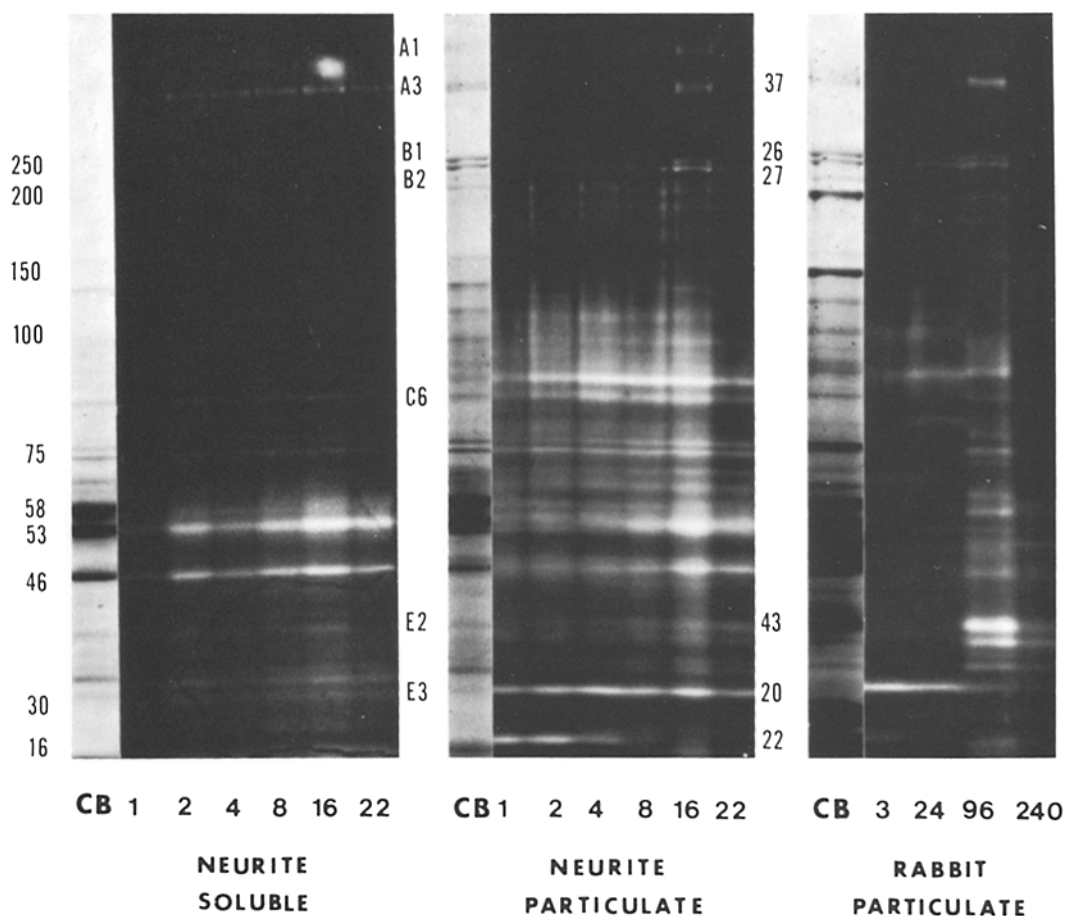


FIGURE 15 Compositional analysis of [ $^{35}\text{S}$ ]methionine-labeled polypeptides undergoing axonal transport. Neurites were labeled by a 1-h pulse period with [ $^{35}\text{S}$ ]methionine ( $50\ \mu\text{Ci}/\text{ml}$ ;  $800\ \text{mCi}/\text{mmol}$ ) followed by incubation with nonradioactive medium containing  $1\ \text{mM}$  methionine. The polypeptides that underwent transport were compared to those of the rabbit retinal ganglion cells by co-electrophoresis on the same gels. The rabbit material was generously supplied by Mr. Pate Skene and Dr. Mark Willard, and its preparation is described elsewhere (61). At 1, 2, 4, 8, 16, and 22 h, the SCG neurites were dissected free of the explants, homogenized, and centrifuged to separate the soluble polypeptides from the particulate ones and prepared for electrophoresis. Numbers at the left refer to apparent molecular weights, in kilodaltons; numbers in the center refer to our nomenclature for the neuritic polypeptides as given in Fig. 13, and the numbers on the right refer to known rabbit polypeptides, as described previously (61). Coomassie blue (CB)-stained gels are included for comparison. The amount of neuritic protein, in micrograms, in each sample was as follows. Neurite soluble: 1 h, 5; 2 h, 11.8; 4 h, 13.5; 8 h, 12.6; 16 h, 12.0; 22 h, 8.7. Neurite particulate: 1 h, 9.7; 2 h, 11.7; 4 h, 13.0; 8 h, 12.4; 16 h, 11.5; 22 h, 10.0.

while particularly useful for the study of general cell types (such as oligodendrocytes; references 49 and 50), present particular problems for the study of specific neuronal types (although rarely some definition of neuronal source may be accomplished, e.g., references 19, 34, and 35). After separation, in many cases, the metabolic and functional capacity of these cells is either short lived or untested.

The use of tissue culture preparations of normal cells, as in the present study, provides the advantages we have outlined earlier. Some concern must be raised, however, regarding the required extensive treatment with antimetabolites. The two main cell types towards which the drugs's actions were aimed were fibroblastic cells and Schwann cells. The action of each of these agents was examined histologically on dorsal root ganglia and

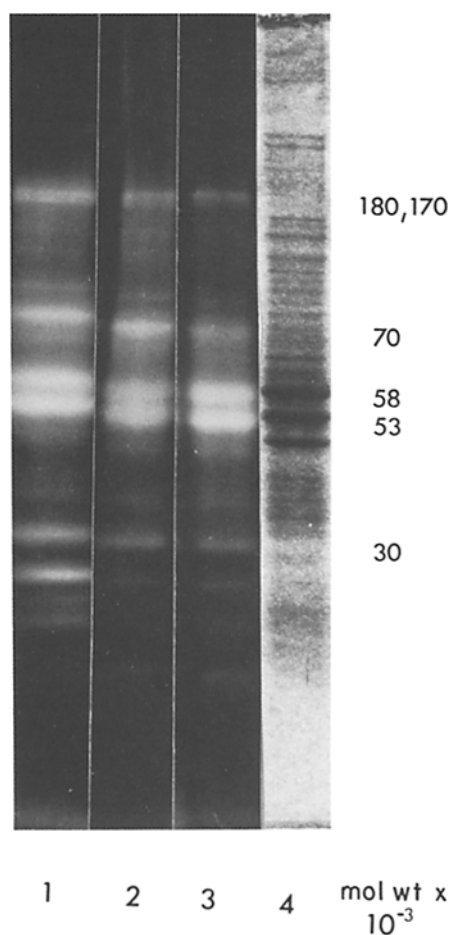


FIGURE 16 External iodination of neurites. Neurites free of non-neuronal cells were removed from SCG explants maintained in vitro for 2 wk, and were iodinated as described in Materials and Methods. After labeling, the neurites were washed, dissolved in electrophoresis buffer, and electrophoresed. The fixed, stained, and dried gels were exposed to Kodak RPR/54 x-ray film to visualize the radioactive bands. Three separate labeling experiments are illustrated in the first three columns, the fourth being a Coomassie blue-stained gel for reference. Apparent mol wts were assigned as described in Materials and Methods.

superior cervical ganglia from perinatal rat pups. In short, ara-C gave the most reproducible killing of all types of non-neuronal cells, and FUDR served to keep the non-neuronal cells from resuming proliferation after ara-C withdrawal. The elimination of supporting cell types from sensory ganglia is more difficult, perhaps due to the potent signal for Schwann cell proliferation present in the sensory axon (65).

The drugs used are both S-phase cytotoxic agents. Flurodeoxyuridine is an inhibitor of thymidylate synthetase (29). Ara-C is known to act at a number of loci during DNA synthesis. It is clear that ara-C is capable of inhibiting DNA polymerase II, the replicative polymerase, from hepatic cell nuclei and that this inhibition can take place at concentrations lower than those necessary

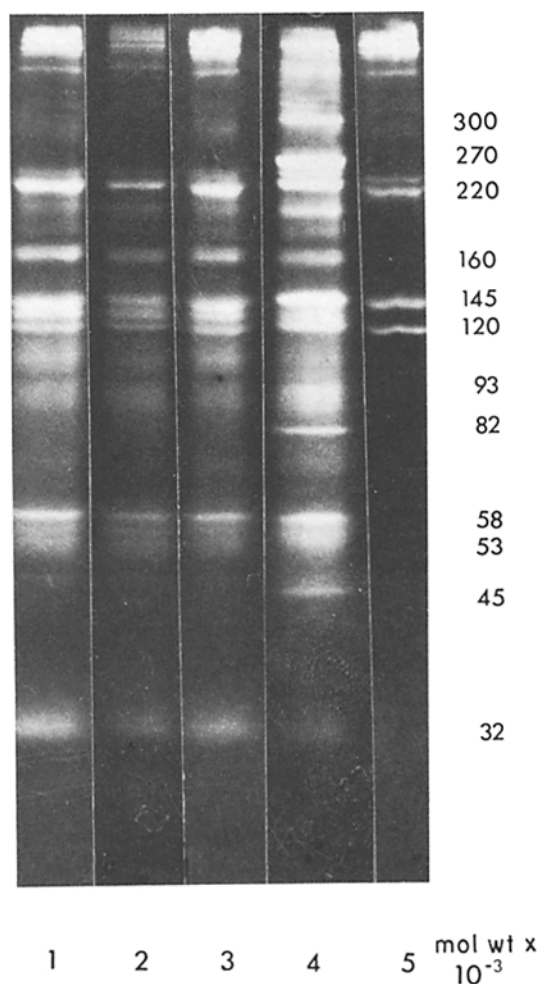


FIGURE 17 External labeling of carbohydrate residues on neurites. Neurites removed from SCG explants maintained in vitro for 2 wk were washed and labeled as described in Materials and Methods. After labeling, the neurites were electrophoresed and the gel was prepared for fluorography. Samples 1-3 above were labeled with NaBT<sub>4</sub> after galactose oxidase treatment of the neurites. Sample 4, neurites labeled with NaBT<sub>4</sub> after periodate oxidation. Sample 5, neurites labeled with NaBT<sub>4</sub> without prior oxidation. The numbers on the right refer to apparent mol wt.

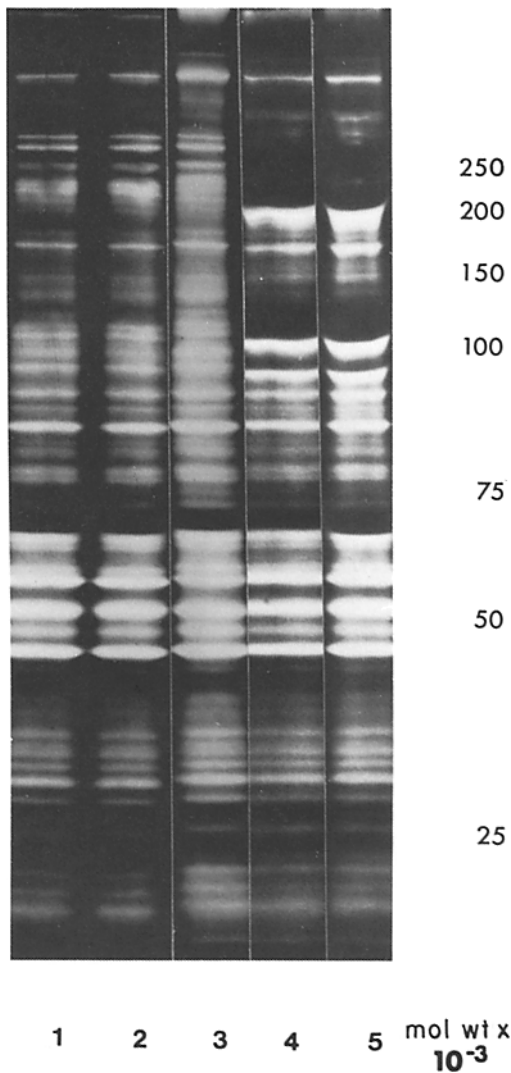


FIGURE 18 SDS gel electrophoretic analysis of [ $^3\text{H}$ ]leucine incorporation into SCG neurons under two culture conditions. Sample 1, neurons maintained in suspension 24 h before labeling; sample 2, neurons maintained in suspension in the presence of  $1\ \mu\text{M}$  colchicine; sample 3, neurons maintained in suspension and labeled in the presence of  $1\ \mu\text{M}$  colchicine; sample 4, neurons plated on collagen and allowed to extend neurites for 24 h; sample 5, neurons plated on collagen in the presence of  $1\ \mu\text{M}$  colchicine which inhibited neurite extension. No qualitative difference could be detected in the polypeptides produced by these cells under culture conditions that either promoted or inhibited neurite elongation. The presence of large amounts of collagen in the plated samples artifactually obscures the molecular weight regions near 100 and 200 kilodaltons in samples 4 and 5.

to effect cell killing in vivo or in vitro (58). Likewise, it has been shown that higher concentrations of the drug ( $10^{-3}\ \text{M}$ ) inhibit the polymerase responsible for DNA repair (15). At even higher concentrations, RNA metabolism is disturbed in chick neural retina cells (32) and hamster fibroblast cells (15). The exact molecular mechanism(s) responsible for cell killing are still not fully understood.

The antimetabolite regimen used in the present study was selected because it removed non-neuronal cells but spared neurons. An important consideration is whether drug effects other than those on DNA synthesis may be present in the neurons. The few studies available report that concentrations of ara-C 50–100 times those used in our study will (a) inhibit RNA synthesis in chick neural retina cells, as mentioned above (32), and (b) will inhibit the incorporation of glucosamine into cells by as much as 85% (28). Under our conditions, neither ara-C nor FUdR has any demonstrable effect on the composition of radioactive proteins synthesized in its presence (Fig. 11). From the reports describing “side effects” of the drugs at levels of  $10^{-3}$  to  $10^{-4}\ \text{M}$ , and from our own studies, discussed above, demonstrating the occurrence of highly differentiated cellular functions in neurons and glia after the cells have been exposed to the drugs at  $10^{-5}\ \text{M}$ , we believe that our method provides neuronal material of normal composition (see also reference 23). Other reports support the view that highly differentiated neuronal functions are fully expressed after extensive treatment with the antimetabolite FUdR (23).

The question must also be raised whether neurons in complete isolation are functionally similar to those growing in the normal companionship of other neurons and glial cells. Media conditioned by target cells or glia have been shown to effect the expression of transmitter-related enzymes by spinal cord (21) or dissociated SCG neurons in culture (47), whereas glial cells will induce the morphological differentiation of neuroblastoma cells (44), and support sensory ganglion neurons in the absence of NGF (11). Compelling evidence has been presented that glial cells surrounding the giant axon of the squid make proteins that are taken into the axoplasm (20, 37, 38). We believe that the long-term maintenance of the autonomic adrenergic neuron in isolation in tissue culture derives from our ability to provide adequate amounts of NGF, the culture medium serving as a

surrogate source of this trophic factor normally obtained by the neuron from target tissues and/or supporting cells in the nerve trunk.

The catalog of functions expressed by the SCG neuron in culture (for general review see references 9, 46, and 48) is now known to include: (a) the maintenance of a normal resting potential and the generation of normal action potentials, (b) the reception of nicotinic cholinergic synapses from neurons within co-cultured spinal cord explants, (c) the synthesis, storage, release, and uptake of norepinephrine, (d) the provision of synapses on smooth, cardiac, and striated muscle, and on fat cells, and (e) the stimulation of Schwann cell proliferation and ensheathment which, as in vivo, does not progress to myelination.

### *Neurite Growth*

A most striking aspect of these cultures is the large amount of neuritic outgrowth that is produced by the explants. This makes possible biochemical studies of this portion of the neuron. After explantation, neuritic outgrowth occurs at a rate of about 300–400  $\mu\text{m}/\text{day}$ . After 2 wk growth, neurite length is commonly 5 mm or greater. This growth rate is similar to those described for both explant type cultures and for elongation of processes from single cells in vitro. Those rates, which vary from  $\sim 200$ – $1,000 \mu\text{m}/\text{day}$  (14, 51, 66), may reflect the substrate upon which the cells are growing (41). The rate of growth of neurites from explants is similar to that described for single processes of sympathetic ganglion neurons (8). A recent study (36) supports our own observations that cultured rat SCG neurons express dendritic morphology (short processes of large diameter) similar to that seen in vivo. This lends support to the notion that the long, thin neuritic processes studied here are representative of growing axons.

From these explants, it is possible to fractionate the neurons into somata and axons, and, by doing so, to investigate the characteristics and functions of these separate cell compartments. From our studies, it is clear that the growing axons mimic in vivo axonal systems investigated in their metabolic dependence on the cell body. The accumulation of radioactive macromolecules in the neurites after exposure of the cells to labeled precursors is dependent on communication between the two. Our studies do not rule out the local modification of macromolecules in the axon as suggested in other studies (3, 4, 67). This may be the explana-

tion for the incorporation of glucosamine seen in the neurites separated from their somata (Table I). Enzymatic assays to fully explore the metabolic capacities of axons as opposed to the neuron cell bodies remain to be carried out.

### *Axonal Polypeptide Transport*

Neurites alone do not exhibit a substantial amount of protein synthesis. Radioactive polypeptides as seen in the axonal transport experiments (Figs. 12, 14, and 15) were transported from the parent somata. We do not yet know how closely the mechanism of transport resembles that seen in axons examined in vivo. However, when an examination of the polypeptides transported into the neurites is made, compositional changes remarkably similar to those described in the rabbit visual system can be demonstrated (Fig. 15). In particular, bands B1 and B2 from SCG cultures co-migrate with bands 26 and 27 from the rabbit ganglion cell axons (62). Band A3 appears slowly in the cultured neurites and, similarly, the analogous band 37 appears in the fourth phase of transport in rabbit. Band 20 in the rabbit, the major fast phase protein, co-migrates with band E3 from the neurites, a major fast-transported protein. Also, while rabbit band 20 and neurite band E3 label heavily with methionine, band 20 remains relatively unlabeled when proline is used as the precursor (M. Willard, personal communication), whereas band E3 is unlabeled in [ $^3\text{H}$ ]leucine-labeled material from culture (Fig. 14), suggesting a relatively high methionine content for this protein in both systems. The similar behavior of a neuritic polypeptide of  $\sim 18,000$  mol wt and band 22 from the rabbit material has already been mentioned. Lastly, band E2 from the neurites co-migrates with the major slow-phase polypeptide, band 43, from rabbits, and mimics that polypeptide in its slow transport velocity.

### *Axonal Surface Compounds*

To date, only the study of synaptosomes has allowed a detailed description of the polypeptide architecture in the neuronal plasma membrane. These particles have been subjected to the same analytical techniques used to analyze the surface of the red cell and the fibroblast. From work in many laboratories, the polypeptide compositions of the plasma membrane of the presynaptic element and the postsynaptic density have been analyzed. In their studies, Wang and Mahler (61)



have identified five polypeptides of 175, 110, 68, 54, and 33 kilodaltons exposed on the neuronal surface membrane. This labeling pattern is remarkably similar to that of the SCG neuron with its main radioiodine-labeled bands of 180–170, 70, 58, 53, and 30 kilodaltons. Polypeptides similar to tubulin have been shown to constitute at least part of the 53 and 58 kilodalton bands from neuritic membranes (16), and they probably correspond to the 54-kilodalton band of the synaptic plasma membrane (61). This band accounts for 25% of the synaptic membrane protein. Further evidence to suggest its identity with the 58 and 53 kilodalton bands of the neuritic membrane includes the observations that both are major fucose-containing glycoproteins from their respective sources (16, 26).

One of the goals of this classification and comparison of axonal polypeptides is the delineation of components that may be distinctive for specific neuronal types. We have pointed out above those axonal components that show similarities in the rabbit retinal ganglion cell axon and the neurite of the rat SCG; these are unlikely candidates for components imparting neuronal specificity. Of the surface components examined, several are generally similar to those of synaptosomal fractions; again, these seem unlikely candidates for markers of separate neuronal species. Until additional types of neurons have been analyzed, it is difficult to identify surface components that are distinctive for SCG neurons. We would, however, single out the component at 45–47 kilodaltons prominently labeled metabolically with fucose and by surface labeling with periodate. There is also a prominent protein in this region in the particulate fraction after amino acid labeling of intact neurites. No similarly prominent polypeptide has been identified as a surface component of synaptosomes or in preparations of retinal ganglion cell axons; it may thus represent a surface moiety distinctive for the SCG neuron.

It is anticipated that further work with neurites of other neuronal types will allow comparison that will delineate components truly unique, in either quantity or quality, to the SCG neuron. The ultimate goal is to be able to assign functions to each of these polypeptides in the complex interplay of plasma membranes that characterizes the developing nervous system.

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## REFERENCES

1. AKESON, R., and H. HERSCHMAN. 1974. Neural antigens of morphologically differentiated neuroblastoma cells. *Nature (Lond.)* **249**:620–623.
2. AMBRON, R. T., J. E. GOLDMAN, and J. H. SCHWARTZ. 1974. Axonal transport of newly synthesized glycoproteins in a single identified neuron of *Aplysia californica*. *J. Cell Biol.* **61**:665–675.
3. AMBRON, R. T., J. E. GOLDMAN, E. B. THOMPSON, and J. H. SCHWARTZ. 1974. Synthesis of glycoproteins in a single identified neuron of *Aplysia californica*. *J. Cell Biol.* **61**:649–664.
4. BARONDES, S. H. 1968. Incorporation of radioactive glucosamine into macromolecules at nerve endings. *J. Neurochem.* **15**:699–706.
5. BOCCHINI, V., and P. U. ANGELETTI. 1969. The nerve growth factor: purification as a 30,000-molecular weight protein. *Proc. Natl. Acad. Sci. U. S. A.* **64**:787–794.
6. BONNER, W. M., and R. A. LASKEY. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83–88.
7. BRAY, D. 1970. Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci. U. S. A.* **65**:905–910.
8. BRAY, D. 1973. Branching patterns of individual sympathetic neurons in culture. *J. Cell Biol.* **56**:702–712.
9. BUNGE, R. P., M. JOHNSON, and C. D. ROSS. 1978. Nature and nurture in the development of the autonomic neuron. *Science (Wash. D. C.)* **199**:1409–1416.
10. BUNGE, R. P., and P. WOOD. 1973. Studies on the transplantation of spinal cord tissue in the rat. I. The development of a culture system for hemisections of embryonic spinal cord. *Brain Res.* **57**:261–276.
11. BURNHAM, P., C. RAIBORN, and S. VARON. 1972. Replacement of nerve growth factor by ganglionic non-neuronal cells for the survival *in vitro* of dissociated ganglionic neurons. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3556–3560.
12. CANCELON, P., and L. M. BEIDLER. 1975. Distribution along the axon and into various subcellular fractions of molecules labeled with <sup>3</sup>H-leucine and rapidly transported in the Garfish olfactory nerve. *Brain Res.* **89**:225–244.
13. CHUN, L. L. Y., and P. H. PATTERSON. 1977. Role of NGF in the development of SCG neurons *in vitro*. *J. Cell Biol.* **75**:694–704.
14. DANIELS, M. P. 1972. Colchicine inhibition of nerve fiber formation, *in vitro*. *J. Cell Biol.* **53**:164–176.
15. EDELSTEIN, M. 1975. A model for the action of ara-c. Ph.D. Thesis. Department of Pathology, Washington University, St. Louis, Mo.
16. ESTRIDGE, M. 1977. Polypeptides similar to the  $\alpha$  and  $\beta$  subunits of tubulin are exposed on the neuronal surface. *Nature (Lond.)* **268**:60–63.
17. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606–2624.
18. FINE, R. E., and D. BRAY. 1971. Actin in growing nerve cells. *Nat. New Biol.* **234**:115–118.
19. FISCHBACH, G. D., D. K. BERG, S. A. COHEN, and E. FRANK. 1975. Enrichment of nerve-muscle synapses in spinal cord-muscle cultures and identification of relative peaks of ACh sensitivity at sites of transmitter release. *Cold Spring Harbor Symp. Quant. Biol.* **30**:347–357.
20. GAINER, H., I. TASAKI, and R. J. LASEK. 1977. Evidence for the glia-neuron protein transfer hypothesis from intracellular perfusion studies of squid giant axons. *J. Cell Biol.* **74**:524–530.
21. GILLER, E. L., J. H. NEALE, P. N. BULLOCK, B. K. SHRIER, and P. G. NELSON. 1977. ChAc transferase activity of spinal cord cultures increased by co-culture with muscle and muscle-conditioned medium. *J. Cell Biol.* **74**:16–29.
22. GLICK, M. C., Y. KIMHI, and U. Z. LITTAUER. 1973. Glycopeptides from surface membranes of neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1682–1687.

23. GODFREY, E. W., P. G. NELSON, B. K. SHRIVER, A. C. BREWER, and B. R. RANSON. 1975. Neurons from fetal rat brain in a new cell culture system: a multidisciplinary approach. *Brain Res.* **90**:1-21.
24. GREENE, L. A., and G. REIN. 1977. Synthesis, storage, and release of acetylcholine by a noradrenergic pheochromocytoma cell line. *Nature (Lond.)*. **268**:349-351.
25. GREENE, L. A., and A. S. TISCHLER. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2424-2428.
26. GURD, J. W., and H. R. MAHLER. 1974. Fractionation of synaptic plasma membrane glycoproteins by lectin affinity chromatography. *Biochemistry*. **13**:5193-5198.
27. HAFKKE, S. C., and N. W. SEEDS. 1975. Neuroblastoma: the *E. coli* of neurobiology. *Life Sci.* **16**:1649-1658.
28. HAWTREY, A. O., T. SCOTT-BURDEN, P. JONES, and G. ROBERTSON. 1973. Inhibition by 1- $\beta$ -D-arabinofuranosylcytosine of the incorporation of N-acetylneuraminic acid into glycolipids and glycoproteins of hamster embryo cells. *Biochem. Biophys. Res. Commun.* **54**:1282-1287.
29. HEIDELBERGER, C. 1965. Fluorinated pyrimidines. *Prog. Nucleic. Acid Res. Mol. Biol.* **4**:1-50.
30. HENDRY, I. A. 1977. Cell division in the developing sympathetic nervous system. *J. Neurocytol.* **6**:299-309.
31. HUBBARD, A. L., and Z. A. COHN. 1972. The enzymatic iodination of the red cell membrane. *J. Cell Biol.* **55**:390-405.
32. JONES, R. E., and A. A. MOSCONA. 1974. Effects of ara-C on differential gene expression in embryonic neural retina. *J. Cell Biol.* **61**:688-700.
33. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680-685.
34. LAM, D. M. K. 1972. Biosynthesis of acetylcholine in turtle photoreceptors. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1987-1991.
35. LAM, D. M. K. 1973. Correction. *Proc. Natl. Acad. Sci. U. S. A.* **70**:284.
36. LANDIS, S. C. 1977. Morphological properties of the dendrites and axons of dissociated rat sympathetic neurons. *Neuroscience*. **3**:525. (Abstr.)
37. LASEK, R. J., H. GAINER, and J. L. BARKER. 1977. Cell to cell transfer of glial proteins to the squid giant axon. The glia-neuron protein transfer hypothesis. *J. Cell Biol.* **74**:501-523.
38. LASEK, R. J., H. GAINER, and R. J. PRZYBYLSKI. 1974. Transfer of newly synthesized proteins from Schwann cells to the squid giant axon. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1188-1192.
39. LASEK, R. J., and P. N. HOFFMAN. 1976. The neuronal cytoskeleton, axonal transport and axonal growth. In *Cell Motility*. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 1021-1049.
40. LAZARUS, K. J., R. A. BRADSHAW, N. R. WEST, and R. P. BUNGE. 1976. Adaptive survival of rat sympathetic neurons cultured without supporting cells or exogenous nerve growth factor. *Brain Res.* **113**:159-164.
41. LUDUBNA, M. A. 1974. Nerve cell differentiation, *in vitro*. *Dev. Biol.* **33**:268-284.
42. MAINS, R. E., and P. PATTERSON. 1973. Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J. Cell Biol.* **59**:329-345.
43. MCCARTHY, K. D., and L. M. PARTLOW. 1976. Preparation of pure neuronal and non-neuronal cultures from embryonic chick sympathetic ganglia: a new method based on both differential cell adhesiveness and the formation of homotypic neuronal aggregates. *Brain Res.* **114**:391-414.
44. MONARD, D., F. SOLOMAN, M. RENTSCH, and R. GYSIN. 1973. Glial induced morphological differentiation in neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1894-1897.
45. OKUN, L. M. 1973. Isolated dorsal root ganglion neurons in culture: cytological maturation and extension of electrically active processes. *J. Neurobiol.* **3**:111-151.
46. O'LAGUE, P. H., P. R. MACLEISH, C. A. NURSE, P. CLAUDE, E. J. FURSPAN, and D. D. POTTER. 1975. Physiological and morphological studies on developing sympathetic neurons in dissociated cell culture. *Cold Spring Harbor Symp. Quant. Biol.* **30**:399-407.
47. PATTERSON, P. H., and L. L. Y. CHUN. 1977. The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. The effects of conditioned medium. *Dev. Biol.* **56**:263-280.
48. PATTERSON, P., L. F. REICHARDT, and L. L. Y. CHUN. 1975. Biochemical studies on the development of primary sympathetic neurons in cell culture. *Cold Spring Harbor Symp. Quant. Biol.* **30**:389-398.
49. PODUSLO, S., H. F. MCFARLAND, and G. M. MCKHANN. 1977. Production of anti-sera to neuronal and oligodendroglial surface components. *Science (Wash. D. C.)*. **197**:270-272.
50. PODUSLO, S. E., and G. M. MCKHANN. 1977. Synthesis of cerebroside by intact oligodendroglia maintained in culture. *Neurosci. Lett.* **5**:159-164.
51. ROISEN, J. F., and R. A. MURPHY. 1973. Neurite development *in vitro*. II. The role of microfilaments and microtubules in dibutyl adenosine 3',5'-cyclic monophosphate and nerve growth factor stimulated maturation. *J. Neurobiol.* **4**:397-412.
52. ROSE, S. P. R. 1976. Functional biochemistry of neurons and glia. *Prog. Brain Res.* **45**:67-82.
53. SATO, G. 1973. *Tissue Culture of the Nervous System*. Plenum Publishing Corp., New York.
54. SCHUBERT, D., S. HEINEMAN, and Y. KIDOKORO. 1977. Cholinergic metabolism and synapse formation by a rat nerve cell line. *Proc. Natl. Acad. Sci. U. S. A.* **74**:2579-2583.
55. SCHUBERT, D., and C. WHITLOCK. 1977. Alteration of cellular adhesion by nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **74**:4055-4058.
56. SHARMA, S. K., W. A. KLEE, and M. NIRENBERG. 1977. Opiate dependent modulation of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3365-3369.
57. STRECK, T. L., and G. DAWSON. 1974. Topographical distribution of complex carbohydrates in the erythrocyte membrane. *J. Biol. Chem.* **249**:2135-2142.
58. STENSTROM, M. L., M. EDELSTEIN, and J. W. GRISHAM. 1974. Effect of ara-CITP on DNA replication and repair in isolated hepatocyte nuclei. *Exp. Cell Res.* **89**:439-442.
59. TRUDING, R., M. L. SHELANSKI, M. P. DANIELS, and P. MORELL. 1974. Comparison of surface membranes isolated from cultured murine neuroblastoma cells in the differentiated or un-differentiated state. *J. Biol. Chem.* **249**:3793-3982.
60. VARON, S., J. NOMURA, and E. M. SHOOTER. 1972. Nerve growth factor proteins. In *Methods and Techniques in Neurosciences*. R. Fried, editor. Marcel Dekker, Inc., N. Y. 203-229.
61. WANG, Y.-J., and H. R. MAHLER. 1976. Topography of the synaptosomal plasma membrane. *J. Cell Biol.* **71**:639-658.
62. WILLARD, M., W. M. COWAN, and P. R. VAGELOS. 1974. The polypeptide composition of intra-axonally transported proteins: evidence for four transport velocities. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2183-2187.
63. WILLARD, M., and K. HULEBAK. 1977. The intra-axonal transport of polypeptide H: evidence for a fifth (very slow) group of transported proteins in the retinal ganglion cells of the rabbit. *Brain Res.* **136**:289-306.
64. WOOD, P. 1976. Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. *Brain Res.* **115**:361-375.
65. WOOD, P., and R. P. BUNGE. 1975. Evidence that sensory axons are mitogenic for Schwann cells. *Nature (Lond.)*. **256**:662-664.
66. YAMADA, K. M., B. S. SPOONER, and N. K. WESSLELS. 1970. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **66**:1206-1212.
67. ZATZ, M., and S. H. BARONDES. 1970. Fucose incorporation into glycoproteins of mouse brain. *J. Neurochem.* **17**:157-163.