DEVELOPMENTAL BIOLOGY 118, 222-232 (1986)

Coordinate Regulation of the Expression of Axonal Proteins by the Axonal Microenvironment

P. SONDEREGGER,*† P. F. LEMKIN,‡ L. E. LIPKIN,‡ AND P. G. NELSON†

*Biochemisches Institut, Universitaet Zurich, Winterthurerstr. 190, CH-8057 Zurich, Switzerland; †Laboratory of Developmental Neurobiology, National Institutes of Child Health and Human Development, National Institutes of Health, Bldg. 36, Rm 2A/21, Bethesda, Maryland 20205; and ‡Image Processing Section, Laboratory of Mathematical Biology, National Cancer Institute, Park Building, Rm 417, Bethesda, Maryland 20205

Received October 15, 1986; accepted in revised form June 16, 1986

The axonal functions that act in the formation of the neuronal network have been shown to occur in close interdependence with the tissue that surrounds the growing axons. However, little is known about the molecular building blocks underlying axonal functions, although more than 400 axonal proteins have been identified. In view of the existence of such a large number of axonal proteins, we have initiated a project to determine the molecules involved in the implementation of particular axonal functions by a selective approach. On the assumption that plasticity in the expression of axonal functions in response to specific features of the local axonal environment may be based on changes in the expression of particular axonal proteins, the axonal proteins of dorsal root ganglion (DRG) neurons were screened for those whose expression responds to environmental influences. DRG neurons were grown in a compartmental cell system that offers separate access to neuronal somas and to their axons and the axons were locally exposed to different populations of cells from the peripheral or central nervous system. The axonal proteins were metabolically labeled and subjected to two-dimensional gel elctrophoresis. Computerized quantitation of the individual axonal proteins revealed that the cocultured cells modulate the synthesis of a few axonal proteins of DRG neurons differentially. The data on the abundance of the newly expressed proteins under varying local environmental conditions were condensed as expression profiles. Comparison of expression profiles and cluster analysis of quantitative gel analysis data revealed that the environmentally modulated proteins subdivide into clusters with common distinct expression profiles under the influence of nonneuronal cells from the peripheral nervous system, nonneuronal cells of the central nervous system, and spinal cord cells, which are composed of neurons and nonneuronal cells. By means of this new, characteristic attribute assigned to environmentally modulated axonal proteins, working hypotheses were made as to their functional role.

INTRODUCTION

The developmental organization of the nervous system as a highly complex network of interacting neurons depends on the capability of neurons to extend their axons over long distances along specified pathways to make specific contact with target neurons. The axonal functions that implement this network formation are phenomenologically described as elongation, sprouting, pathfinding, fasciculation, synthesis of a specific neurotransmitter, and synapse formation. However, the neuronal mechanisms implementing and regulating axonal behavior are still very poorly understood in molecular terms. In previous studies on axonal proteins (Sonderegger *et al.*, 1983; 1984) we have shown that more than 400 axonal proteins are detectable in axons of dorsal root ganglion neurons by two-dimensional gel electrophoresis and more than 1000 proteins were present on more heavily loaded gels (P.S., unpublished). Confronted with such an overwhelming molecular complexity it seems obvious that, at the present time, the identification of the proteins involved in a particular axonal function cannot be approached in a random way. However, the fact that most of the axonal functions recognized thus far have been found to be subject to environmental control of some sort (Ramon y Cajal, 1928; Levi-Montalcini *et al.*, 1954; Sperry, 1963; Letourneau, 1975; Patterson, 1978; Gundersen and Barrett, 1979; Mudge, 1984) offers opportunities for a selective approach. On the working assumption that the molecular basis of these environmentally regulated axonal functions consists of, at least in part, environmentally regulated proteins, one may screen the detectable axonal proteins for those that are subject to environmental modulation in their expression. In a series of *in vivo* experiments performed under this rationale, a number of proteins, termed "growth-associated proteins", have recently been identified. Nerve crush experiments on central and peripheral nerves and in a variety of amniotic and anamniotic animals revealed that a number of axonally transported proteins only occurred in situations where axonal regeneration was in action (for a review cf. Skene, 1984).

We chose an *in vitro* approach to the understanding of axonal functions at the molecular level in order to be able to expose axons locally to a wide variety of controlled experimental conditions and to be able to observe

0012-1606/86 $3.00

their behavior more closely (Fischbach and Nelson, 1976). An experimental model was developed for selective study of proteins that are axonally transported and either incorporated into axons or secreted (Sonderegger *et al.*, 1983, 1984). As a suitable neuronal cell type we chose dorsal root ganglion (DRG)[1] neurons. DRG neurons are a distinct, although not homogenous, population of cells. The branch of the axon growing out from the neuronal soma in the DRG towards the spinal cord, first is entirely surrounded by a peripheral glial environment, participating in one single fascicle within the dorsal root. Upon entering the spinal cord at the transitional fringe, the axons of the dorsal root come into contact with central glia and split to reach their various destinations. Upon meeting spinal cord neurons, they decide whether or not to stop growing and make a synapse. After injury of dorsal roots, the axons of DRG neurons regenerate readily as long as they are in a peripheral glial environment but virtually stop elongation at the transition from the peripheral to the central nervous system (Perkins *et al.*, 1980; Stensaas *et al.*, 1979; Reier *et al.*, 1983). Obviously, a number of behavioral features of these axons are under the control of the axons' local environment. DRG neurons can also be dissected with relative ease, can be well maintained in culture, and respond in a highly differentiated manner to environmental stimuli. On cultured DRG neurons, environmental influences are found to act on axon elongation (Levi-Montalcini *et al.*, 1954; Baron van Evercooren *et al.*, 1982; Edgar *et al.*, 1984), the guidance of the axonal pathway (Gundersen and Barrett, 1979; Letourneau, 1975), the choice of the neurotransmitter (Mudge, 1981), and the morphology of the axons' initial segment (Mudge, 1984). These facts make DRG neurons a particularly rich model for the study of the environmental regulation of axonal functions and the intriguing behavioral differences of axons during development and repair from injury in the central and peripheral nervous system of amniotic vertebrates.

When chicken embryonic DRG neurons are cultured in the center chamber of the compartmental cell culture system devised by Campenot (1977, 1979), their axons cross beneath a barrier into the side compartments whereas the cell somas are retained in the center compartment. By offering separate access to neuronal cell somas and to their axons, this system allows the study of the expression of axonal proteins selectively even in axons that are embedded in a complex cellular environment (Sonderegger *et al.*, 1983). Hence, this system offers unique possibilities for the investigation of the modulating influences of the local axonal microenvironment on the expression of axonal proteins and, hence, on axonal function. In a previous paper, we reported on specific changes in the expression of axonal proteins induced by peripheral and central nonneuronal, glia-type cells (Sonderegger *et al.*, 1985). In the present study, the environmentally induced changes exerted on the expression of axonal proteins of DRG neurons by local coculture of the axons with different cell types were quantified and compared. DRG neurons were grown in the compartmental cell culture system. The axons extending into the side compartments were exposed locally to different environmental conditions by local coculture with peripheral nonneuronal cells (Fig. 1d), central nonneuronal cells (Fig. 1e), and a complete population of spinal cord cells containing neurons and glia (Fig. 1f). The cellular and humoral environment around the DRG cell somas and the proximal area of the axons was kept constant. The newly synthesized proteins were then metabolically labeled with [^{35}S]methionine, added to the neuronal somas in the center compartment. The cellular material of the side (axonal) compartments was harvested and the side compartment proteins were subjected to two-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (O'Farrell, 1975) followed by fluorography (Bonner and Laskey, 1974). Proteins synthesized and transported into axons under different environmental conditions were quantified and compared using GELLAB, a system for computerized gel image analysis (Lipkin and Lemkin, 1980; Lemkin *et al.*, 1982, 1984; Lemkin and Lipkin, 1983a,b). The proteins with changed expression in any of the experimental classes were submitted to cluster analysis of their expression profiles (Anderberg, 1973; Hartigan, 1975).

MATERIALS AND METHODS

Compartmental Cell Cultures

The compartmental cell culture system (Campenot, 1977, 1979) was used in a version modified for selective metabolic labeling of axonal proteins (Sonderegger *et al.*, 1983, 1984, 1985). All cell culture conditions were exactly as described previously (Sonderegger *et al.*, 1983, 1985). Formation of synapses between DRG axons and cocultured spinal cord neurons has been demonstrated by intracellular recordings (Sonderegger *et al.*, 1983). The cellular composition of the nonneuronal cultures from the peripheral and central nervous system has been assessed by fluorescent-cell marker studies (Sonderegger *et al.*, 1985).

[1] Abbreviations used: DRG, dorsal root ganglion; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DRGNN, peripheral nonneuronal cells from dorsal root ganglia; SCNN, central nonneuronal cells from spinal cord; VSC, cells from the ventral half of the spinal cord.

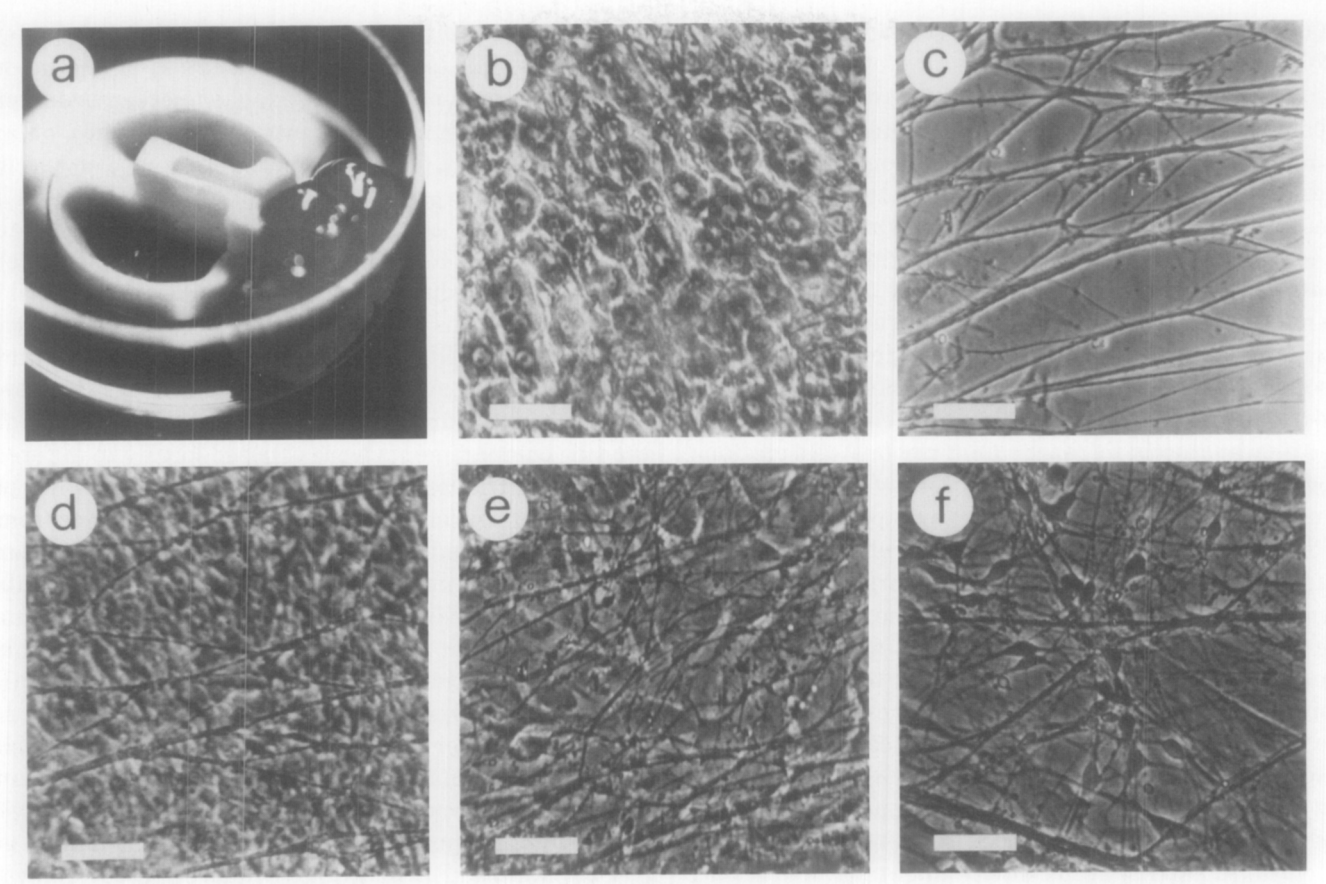

FIG. 1. Morphological aspects of axons of DRG neurons grown in different environments. (a) The compartmental cell culture system as devised by Campenot and modified for metabolic labeling of axonal proteins by Sonderegger *et al.* (b) DRG neurons and glia in the center compartment. (phase-contrast optics). (c) Axon fascicles in the side compartment of the compartmental cell culture system, 10 days after plating the dissociated DRG cells in the center compartment (phase-contrast optics). (d) Cocultured peripheral nonneuronal cells in the side compartments together with the axons of the DRG neurons. (e) Cocultured central nonneuronal cells from spinal cord in the side compartments together with axons of the DRG neurons. (f) Cocultured ventral spinal cord cells in the side compartment together with axons of the DRG neurons. Synapse formation between DRG axons and ventral spinal cord neurons in this system has previously been reported (Sonderegger *et al.*, 1983). (a) ×1.7; (b–f) ×220; bar = 45 μm.

Selective Metabolic Labeling of Axonal Proteins and Two-Dimensional Gel Electrophoresis

Selective metabolic labeling was done as described previously (Sonderegger *et al.*, 1983, 1984, 1985). The newly synthesized proteins were labeled by addition to the center compartment of labeling medium composed of methionine-free growth medium substituted with 15 μ*M* unlabeled methionine, and 1 mCi/ml [^{35}S]methionine (approx. 1,000 Ci/mmole, New England Nuclear Corp., Boston, Mass.). The medium of the side compartments was identical, except that 4 m*M* unlabeled methionine replaced the radioactive methionine. Incubations were for 40 hr to allow for accumulation of the proteins of all axonal transport rate classes (Wilson and Stone, 1979). After labeling, the axons in the side compartments were washed twice with Dulbecco's PBS (GIBCO Laboratories). The cellular material was dissolved in 2% SDS and 5% β-mercaptoethanol at a temperature of 90°C, collected, pooled, and processed for two-dimensional electrophoresis. Two-dimensional SDS-PAGE was done essentially as developed by O'Farrell (1975) and has been described in detail (Sonderegger *et al.*, 1985). The selectivity of this procedure for labeling axonal proteins exclusively has been demonstrated and extensively discussed previously (Sonderegger *et al.*, 1983, 1984).

With a labeling time of 40 hr, the radioactivity of a given protein is likely to be a measure of its steady state abundance, if its axonal transport rate is high. The proteins transported at slower rates, however, may not have reached steady state abundance yet (for transport rates cf. Lorenz and Willard, 1978). Under all circumstances, however, the radioactivity in a given spot is a complex function of the rates of synthesis, axonal transport, posttranslational modification, and degradation, referred to as "protein expression", for the sake of brevity.

Quantitative Analysis of Two-Dimensional Electropherograms

The GELLAB system for the computerized analysis of two-dimensional SDS-PAGE was used to detect protein differences between axons exposed to different environmental conditions (Lipkin and Lemkin, 1980; Lemkin *et al.*, 1982, 1984; Lemkin and Lipkin, 1983a,b). This system acquires data by scanning fluorographic replicas of the two-dimensional electropherograms, along with a neutral density calibration wedge, by a high resolution black-and-white TV camera which digitizes them into 512 × 512 picture element images with 256 gray values (white to black). The images are subsequently calibrated in terms of optical density units and thereafter all measurements are in terms of integrated optical density. GELLAB then segments individual spots in each gel image. The user selects a reference gel (denoted the Rgel) from this set of N gels and interactively defines a small number of landmark spots. Corresponding landmark spots are then manually identified on each of the remaining N-1 gels. Pairing of the remaining spots between the different gels can subsequently be performed automatically. Corresponding spots from different gels are eventually merged into a composite gel data base and are called Rspot sets. They consist of those spots in the N gels which correspond to the same spot in the Rgel. In the present study, the data were normalized by the least-squares method (Lemkin and Lipkin, 1983a). The N gels were subdivided into subsets of gels of the same experimental condition. These subsets are called experimental classes in GELLAB. Statistical comparisons were conducted on experimental class data containing at least five gels/class obtained by independent experiments using F tests at confidence limits of 0.90 and 0.95. The CGELP program in GELLAB has facilities for interactively searching the composite spot data base as well as saving the results of searches and visualizing them with Rmaps, i.e., a copy of any of the original gel images with selected Rspots labeled. (Examples of Rmaps are shown in Fig. 5).

The CGELP program in GELLAB provides the facility to generate so-called mosaic images. A mosaic image is an image composed of panels of subregions of the gel images surrounding a particular spot ordered by increasing normalized density. The assumed relative coordinates of the center of the spot to be investigated are used to position the center of each panel and are labeled with a white dot. Each panel represents a different gel and 16 gels can be displayed in one mosaic image. The spots of the surrounding region give a morphological context which allows one to verify that the spot indicated is in fact the one under investigation. Hence, as mispaired spots are easily detected by visual inspection in these mosaic images, this facility provides an excellent tool for the control of accurate spot pairing. We made extensive use of the mosaic image facility in checking for correct pairing of every spot that was found to be significantly changed in any of the six permutations of pairwise comparisons between experimental classes.

Cluster Analysis of the Modulated Proteins

Given a set of polypeptide spots which differ significantly between two or more experimental classes, one might wish to find whether these spots group into subsets with similar expression profile. By similar expression profile we mean here that if one polypeptide increases (or decreases) in concentration under different experimental conditions then spots of the same subset (i.e., similar expression profile) will change correspondingly. Such subsets of proteins may contain candidates for proteins that are coregulated. Coregulated proteins might be considered candidates for proteins involved in the same or related cellular functions.

To screen for proteins with similar expression profiles under a variety of local influences imposed upon the axons, histogram-type graphs were plotted, taking mean normalized density as the ordinate (Fig. 3). By visual inspection, the 13 modulated proteins were subdivided into clusters with similar expression profiles. However, the manual approximation of the clustering of the expression profiles, with increasing number of spots (objects) and data from different experimental conditions (features), becomes increasingly difficult. In addition, an estimate for the closeness of the clustering would be desirable, which would be very hard to obtain by hand. A number of techniques are available from the field of cluster analysis (Anderberg, 1973; Hartigan, 1975) which attempt to uncover such similarity and an application of such techniques to two-dimensional gel data has recently been reported (Anderson *et al.*, 1984).

A clustering algorithm, in general terms, is applied to feature data of N objects each having values for K features. In the case of two-dimensional-gel spot data, a set of spots (objects) may be searched for cluster formation considering their features displayed under different experimental conditions. The features considered may be (a) the mean values of the concentration in different experimental classes, (b) the ratios of the mean densities of the same spot in pairs of experimental classes for all permutations of classes, or (c) the combination of (a) and (b). Given N objects (by default the total number of different spots that changed under different experimental conditions), the various cluster analysis techniques basically try to iteratively find the

next most similar clusters. This group of two existing clusters is then defined to be a new cluster. Complete clustering is done by repeating this process N-1 times to finally form one cluster from N initial objects. The particular metric definition of "similar" that we use is the K-dimensional Euclidian distance between any two clusters being compared. Each of the K features is autoscaled to a standard deviate scale by subtracting its mean and dividing by its standard deviation, computed over the N samples. This permits a uniform comparison of features. Spencer (1984) estimates the K-dimensional vector for new clusters using a group-weighted average. After generating the N-1 clusters at various levels it is useful to visualize them. This may be done by use of dendrograms, the Delaunay triangulation, Voronoi diagram, etc. (Shapiro, 1983). Such visualization facilitates interpretation of the clusters in the context of the biological problem. The dendrogram display is used here as it was produced automatically as part of the algorithm we adopted (Spencer, 1984).

As clusters are ranked by their minimum distance between the objects which form them, distinct subsets among the clustered proteins were discernable. If an adequate threshold value for this minimum distance could be estimated, then significant (in a statistical sense) clusters could be noted. Such "stopping" rules" have been proposed (Mojena, 1975), which treat the intercluster distances as a distribution and then performs a statistical test on that distribution. A modification of that technique is used here taking the approximate intercluster distance cut-off criteria of "significant clusters" as being those whose cluster similarity distance is less than the mean cluster similarity distance plus one standard deviate.

RESULTS

The Environmentally Modulated Axonal Proteins Subdivide into Clusters with Similar Expression Profiles

Under the conditions employed for these experiments, more than 400 protein spots were discernable on each gel. Computerized quantification of the individual protein spots using GELLAB revealed that the bulk of the axonal proteins were expressed at identical relative quantities under all four experimental conditions. Thirteen proteins, however, were significantly changed in at least one of the six possible pairwise comparisons of the four experimental classes. Figure 2 illustrates two of these environmentally modulated proteins, viz., spots 85 and 90, throughout the four experimental classes. The ratios of the mean least-squares-normalized densities of these pairwise comparisons are listed in Table I and range from 0.15 to 7.69. The mean normalized densities of these protein spots under all experimental conditions are plotted in Fig. 3. Proteins 85 and 86 were previously reported increased and protein 435 was decreased under coculture of DRG axons with spinal cord cells under conditions where synapse formation occurred (Sonderegger, 1983). The fourth of the changed proteins of that previous study, an acidic protein with a molecular weight of about 65K, is not included in the present analysis, because of saturation on the X-ray films under standard fluorographic exposure.

To test whether the environmentally modulated proteins showed any common traits, their relative abundance under different environmental conditions was considered (Fig. 3). The order of the environmental conditions in this histogram-type graphic representation was chosen to simulate the order an outgrowing axon from a DRG neuron would be expected to encounter. However, no claim is made, that the experimental paradigm used is reflecting the *in vivo* situation during development or regeneration accurately. Furthermore, the interpretation of the expression profiles does not depend on the order of experimental conditions chosen for the graph.

As can be seen in Fig. 3, the modulated proteins are roughly subdividable into four clusters. Cluster I contains proteins characterized by high abundance in the absence of any cocultured cells. Coculture of any of the three populations reduced these proteins markedly. Cluster II contains five proteins, with high abundance under coculture with peripheral nonneuronal cells as the most striking feature. Cluster III is composed of two proteins that suffered a marked depression by coculture with central nonneuronal cells. Cluster IV was composed of two proteins highly abundant in central nonneuronal cells and spinal cord cells, but which otherwise did not seem to behave similarly throughout the various conditions. However, these clusters, although exhibiting common traits, seemed to be heterogeneous in other features. The modulated proteins were subjected to cluster analysis, a technique designed to subdivide objects into groups containing members which are considered to be similar (in some abstract feature space) (Anderberg, 1973; Hartigan, 1975). The results (Fig. 4) confirmed the subdivision of the 13 proteins into four major clusters, as was obvious from visual inspection of the expression profiles (Fig. 3). In addition, the capability of the clustering algorithm to give a quantitative estimate of the similarity of the objects grouped together showed that second-order quantitative differences within the clusters occurred. Two of the "coculture-depressed proteins" (cluster I), namely 516 and 335, are

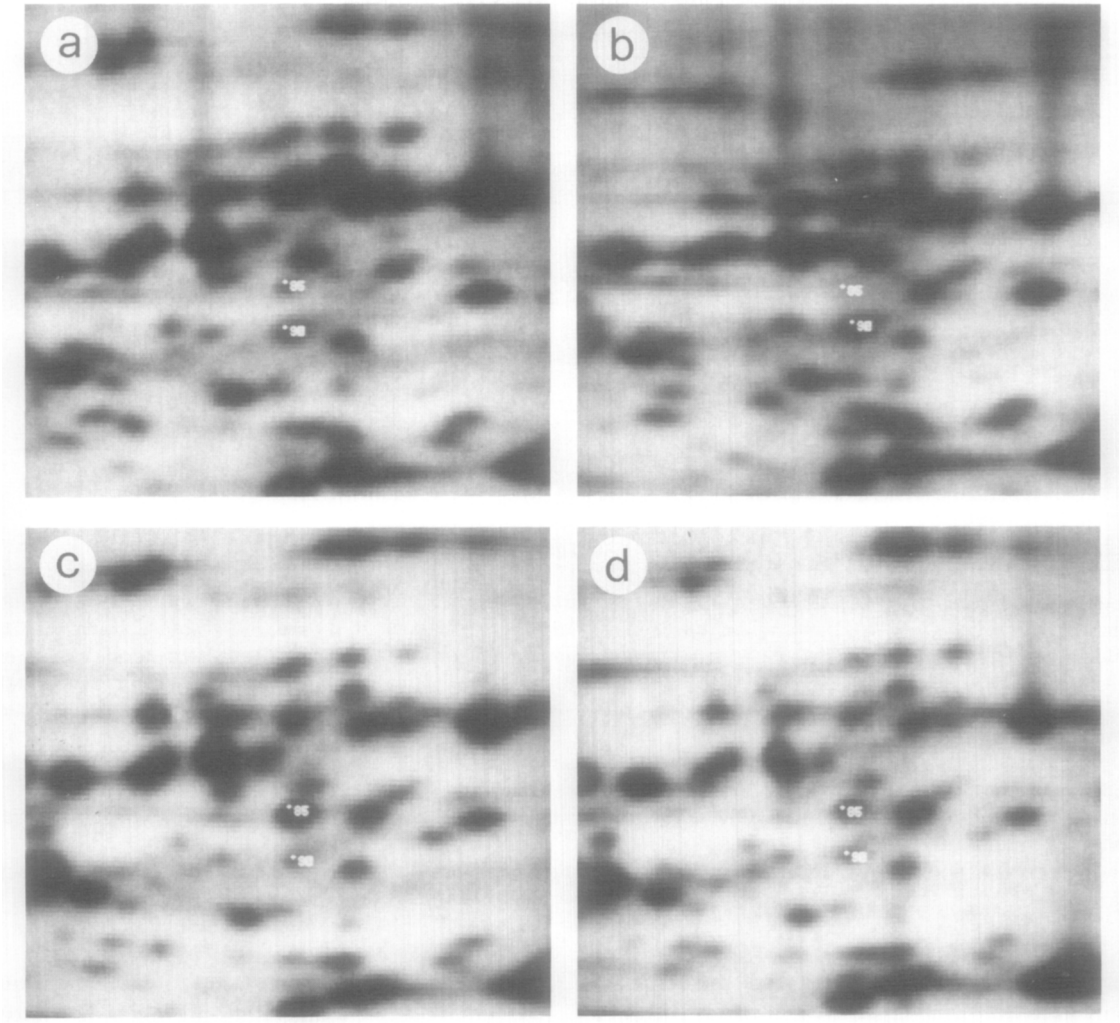

FIG. 2. Illustration of two environmentally modulated proteins throughout all four experimental conditions. Four-times zoomed images. The location of the protein spot 85 and 90, respectively, with respect to p*I* and apparent molecular weight can be taken from Fig. 5. (a) Proteins of axons grown without coculture. (b) Proteins of axons grown in the presence of nonneuronal cells from the peripheral nervous system. (c) Proteins of axons grown in the presence of nonneuronal cells of the central nervous system. (d) Proteins of axons grown in the presence of ventral spinal cord cells.

grouped closely together and clearly separated from spot 435 and even further distant from spot 582 in the abstract feature space. Similarly four of the "peripheral glia-enhanced proteins" (cluster II), namely spots 359, 160, 484, and 90, are grouped very closely together and are clearly distinct from spot 162. The pairs of proteins grouped together as "central glia-depressed proteins" (cluster III) and "central nervous system cells-enhanced proteins" (cluster IV) were revealed as closest to each other, but in both pairs the cluster similarity distance is well beyond the cut-off distance. The further significant clustering of cluster I and cluster II may also indicate a relationship between these two groups of proteins.

DISCUSSION

Progress in the analytical technologies for the detection and isolation of proteins has made it possible to separate and display the proteins from complex biological tissues. In particular, the technique of two-dimensional SDS-PAGE (O'Farrell, 1975) allows us to identify and characterize large numbers of individual proteins from heterogeneous cellular material. Theoretically, between 7,000 (O'Farrell, 1975) and 10,000 (Anderson *et al.*, 1984) individual proteins have been estimated to be resolvable on a two-dimensional gel. At the same time, recombinant DNA technologies are providing data on impressive numbers of genes and gene products. As an

TABLE 1
COMPARISON OF THE AMOUNTS OF AXONAL PROTEINS EXPRESSED UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Spot no.	MW	Cocultures compared (ratio of mean normalized densities)					
		DRGNN vs none	SCNN vs none	VSC vs none	SCNN vs DRGNN	VSC vs DRGNN	VSC vs SCNN
85	65K	(0.59)	(3.69)	3.70‡	6.67*	6.18‡	(0.99)
86	65K	3.83‡	7.21‡	7.69‡	1.89*	2.06‡	(1.09)
90	60K	(1.76)	(0.73)	(0.46)	0.42‡	0.26‡	0.63*
160	97K	(1.45)	(0.81)	(0.67)	0.56*	0.46‡	(0.83)
162	97K	1.84‡	(1.16)	(1.55)	0.63‡	0.85‡	(1.34)
335	48K	0.60‡	(0.65)	0.64‡	(1.08)	(1.7)	(0.98)
359	37K	(1.79)	(0.90)	(0.72)	0.51‡	(0.40)	(0.80)
361	35K	(0.82)	0.32*	(1.23)	0.39*	(1.49)	3.85*
435	37K	0.51*	0.38*	(0.68)	(0.74)	(1.33)	(1.79)
462	13K	(1.14)	0.17*	(0.77)	0.15‡	(0.68)	4.76*
484	27K	(1.30)	(0.63)	0.50*	0.48*	0.39‡	(0.80)
516	31K	0.61‡	(0.63)	0.48‡	(1.04)	(0.79)	(0.76)
582	34K	0.29‡	0.21‡	0.33‡	(0.73)	(1.12)	(1.54)

Note. Ratios of the mean normalized density values of corresponding proteins that have been found significantly different in at least one of the comparisons of the four experimental classes of coculture, namely DRG axons without cocultured cells (none), DRG axons with cocultured peripheral nonneuronal cells (DRGNN), DRG axons with cocultured central nonneuronal cells (SCNN), and DRG axons with cocultured ventral spinal cord cells (VSC). The second column (MW) indicates the apparent molecular weight as determined by the protein spots relative mobility with respect to the molecular weight markers. (cf. Material and Methods). Part of the data shown here is based on the raw data of previously reported experiments (Sonderegger *et al.*, 1983, 1985).

* Statistically significant difference of the normalized density values for a protein spot occurred between two experimental classes (F test, $n = 5$) at a confidence limit of 0.90.

‡ Statistically significant difference of the normalized density values for a protein spot occurred between two experimental classes (F test, $n = 5$) at a confidence limit of 0.95.

If no difference was revealed by either statistical test, the ratio of the mean normalized densities of the compared experimental classes is put between parentheses.

example, an estimated number of 30,000 mRNAs are found in the brain of the rat (for a review cf. Sutcliffe and Milner, 1984). The large number of cellular proteins that can be detected emphasizes our ignorance regarding the function of the majority of the individual proteins. As opposed to the relative ease of the process of identification of novel proteins, the function of a particular protein is much harder to demonstrate. The assignment of functions to proteins has obviously become the most difficult step in the elucidation of the mechanisms nature employs in the implementation of biological functions and certainly is one of the challenges of the future research in biology (for a discussion of related issues cf. Barnstable *et al.*, 1983; Newmark, 1983).

The present paper reports on the first steps in an attempt to select functionally intriguing axonal proteins for further studies. The expression of axonal proteins was selectively studied by metabolic labeling in a compartmental cell culture system that allows separate access to neuronal somas and their axons. Axonal proteins whose expression was modulated by the axon's local cellular environment were compared with respect to their relative abundance under different environmental conditions. By their expression profiles, they roughly subdivided into four clusters. Computerized cluster analysis confirmed the close relationship between the manually grouped expression profiles and, in addition, revealed distinct subclusters of proteins within the four manually estimated clusters.

Each of the local coculture conditions evoked characteristic phenomenological responses of the axons. Coculture with central nonneuronal cells led to a finer branching pattern than that observed in axons growing on a collagenized culture dish or axons growing together with peripheral nonneuronal cells (Fig.1 and Sonderegger *et al.*, 1985), and, in the presence of target neurons (coculture with spinal cord cells), the DRG axons established synaptic contacts (Sonderegger *et al.*, 1983). Hence, it is tempting to think that proteins modulated in their expression by the axons' local environment represent elements subserving various concomitantly modulated axonal functions and to try to obtain clues for the functional role of the environmentally modulated proteins by correlation of their expression profiles with the profiles of activity of certain axonal functions.

The expression profiles of five proteins display, as their

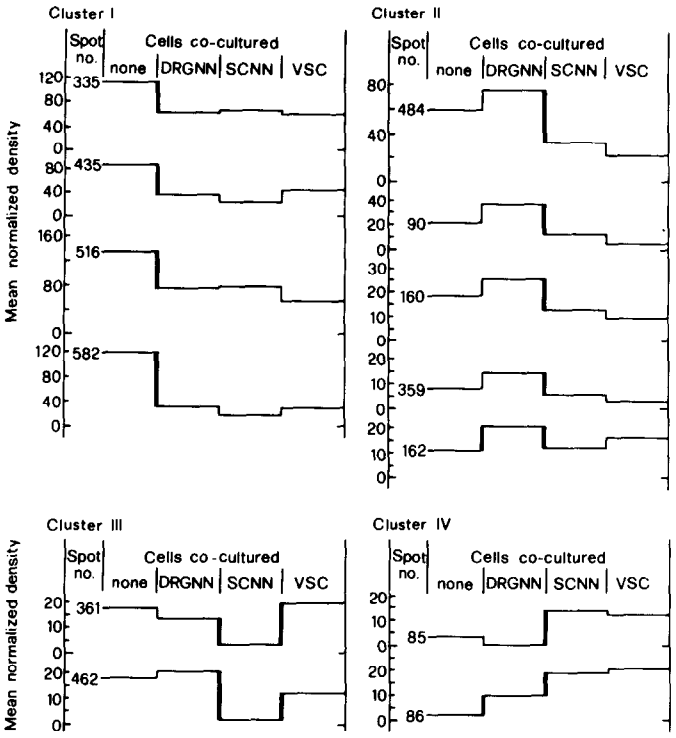

FIG. 3. Expression profiles of the 13 proteins previously determined to be modulated by environmental influences. Histogram-type plots of mean normalized density versus coculture condition. The thick vertical bars indicate where statistically significant differences in the comparison of the adjacent experimental classes occur. The expression profiles were manually clustered according to their most prominent feature. Heterogeneity of these groups is obvious at more detailed inspection and has been dealt with by computerized cluster analysis (see Fig. 4). (a) Cluster I proteins: proteins with high relative abundance in the absence of any cocultured cells (none). (b) Cluster II proteins: proteins with high relative abundance under local coculture with peripheral nonneuronal cells (DRGNN). (c) Cluster III proteins: proteins with depressed relative abundance under coculture with central nonneuronal cells (SCNN). (d) Cluster IV proteins: proteins with high relative abundance under local coculture with central nervous system cells (SCNN and VSC).

most prominent feature, a marked increase of their expression under local influence of peripheral glia (cluster II proteins). We suggest that these proteins could be involved in some function conferring on the axons the linear outgrowth pattern they display while in the peripheral nervous system, on their way to the peripheral sensory organs as well as on their way to the point where they enter the central nervous system. Analogously reasoned, the increased expression of two proteins under the influence of cells of the central nervous system (cluster IV proteins) may suggest that they are involved in the acquisition of the highly branched growth pattern the DRG axons take on after their entry into the central nervous system.

The fact that the proteins of cluster III are expressed under all conditions except coculture with central glia suggests that a component of central glia is exerting a depressing activity on the expression of these proteins rather than all other coculture conditions, including the controls without cells added, exerting a stimulatory effect. The cocultured central nonneuronal cells from spinal cord (SCNN) were mainly composed of glia cells and neurons were absent, whereas the cocultures termed ventral spinal cord cells (VSC) contained neurons in addition to glia cells. This suggests that the presence of neurons counteracts the suppressing activity of the central glia cells for these two proteins. It is intriguing to think that this effect may be directly evoked by neurons, possibly in conferring on the axons the capacity for synapse formation.

A relatively large proportion of the modulated proteins is suppressed by all coculture conditions with respect to the axons grown alone (cluster I proteins). A possibly analogous situation has recently been reported in that the extracellular matrix protein laminin has been found expressed by astrocytes in culture (Liesi *et al.*, 1983), whereas astrocytes *in situ* have not been found to

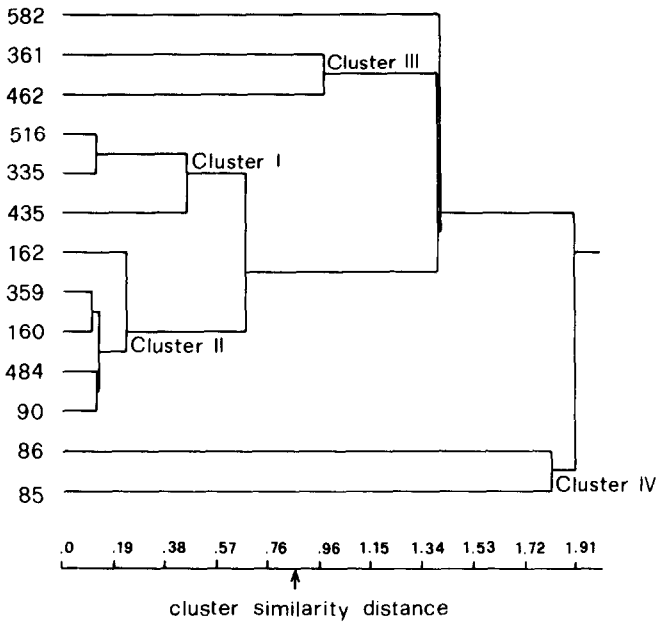

FIG. 4. Dendogram representing the clustering pattern of the 13 environmentally modulated proteins. Computerized cluster analysis was performed with the 13 modulated spots as the objects and the ratios of the mean values of all permutations of pairwise comparisons of all experimental classes as the features (data of Table I). The numbers to the left of the tree indicate the two-dimensional-gel spot numbers. The abscissa represents cluster similarity distance as measured in standard error distance. As a cutoff criterion of "significant clusters" the mean cluster similarity distance plus one standard deviate was used (cutoff indicated by vertical arrow).

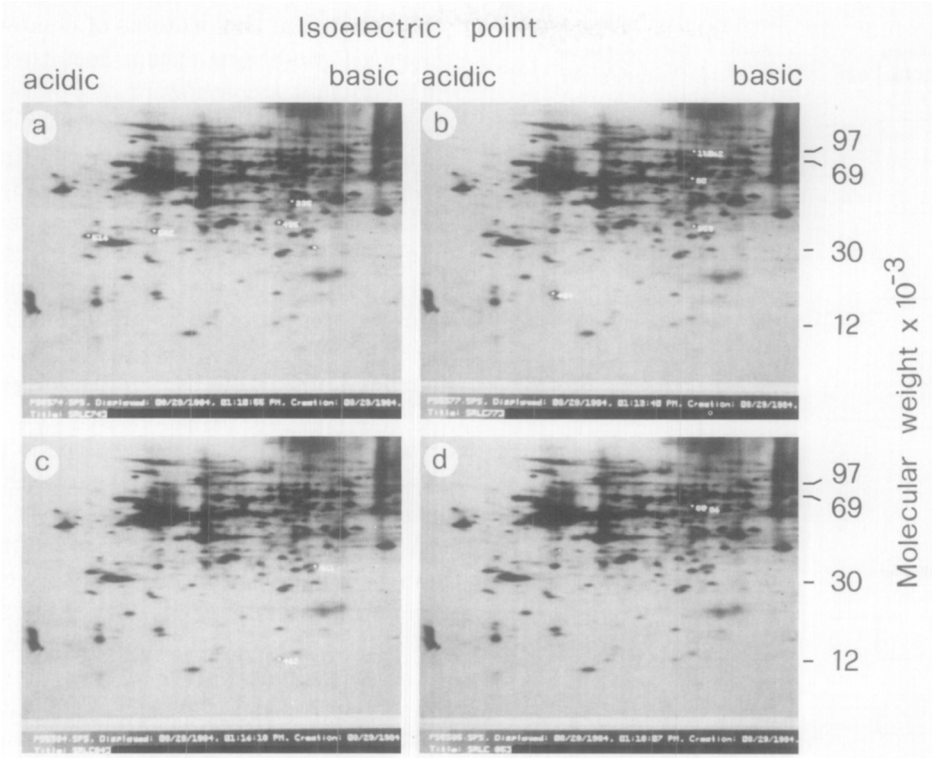

FIG. 5. Digitized images of two-dimensional electropherograms indicating the position of those proteins with similar expression profiles (all panels 50% reduced). The same gel picture is used for all panels. (a) Cluster I proteins: proteins with high relative abundance in the absence of any cocultured cells. (b) Cluster II proteins: proteins with high relative abundance under local coculture with peripheral nonneuronal cells. (c) Cluster III proteins: proteins with depressed relative abundance under coculture with central nonneuronal cells. (d) Cluster IV proteins: proteins with high relative abundance under local coculture with central nervous system cells.

express laminin except possibly during central nervous system development (Liesi, 1985). Suppression by all employed coculture conditions could mean that these proteins function very early during axon outgrowth, when peripheral axons may be in contact with nonneural cells or glial precursor cells that have not been included in the present coculture study. Clues to the functions these four proteins could be involved in would possibly come from the inclusion of such cell types for coculture.

Modulation of an axonal function may be controlled by variation of the supply of the necessary molecular building blocks by the neuronal soma, or by functional modification of existing building blocks by regulatory events. The number of the proteins with changed relative expression in association with local changes of the axonal environment is relatively small. The major part of the axonal proteins is expressed at identical proportions. This finding is in accordance with *in vivo* studies on axon regeneration after nerve crush (Hall *et al.*, 1978; Perry and Wilson, 1981; Skene and Willard, 1981). Since a number of subfunctions are comprised in axonal events such as elongation or synapse formation, it is unlikely that this small number of proteins responding to changes in the local axonal environment represents the entirety of the molecular building blocks subserving concomitantly changing axonal functions. As previously pointed out in the context of "growth-associated proteins" (Levine *et al.*, 1981; Skene, 1984), such a constellation suggests that the neuron normally supplies the proteins required for the implementation of, at least part of, the momentarily inactive axonal functions (Perry and Wilson, 1981) and that activation or inactivation of such functions may be controlled by a small number of environmentally regulated gene products. This view is supported by phenomenological evidence that two important axonal functions, viz., nerve sprouting and synapse formation, can be activated in distal axon stumps after their separation from their somas (Rotshenker, 1981). Similarly, short latency axonal responses to nerve growth factor, such as filopodial motility and growth cone locomotion, were found to be implemented locally and independent of the cell body and in the absence of RNA and protein synthesis (for a review cf. Greene, 1984).

The expression profile under defined local environmental influences to which the axons are exposed, adds a new, characteristic attribute to an environmentally

modulated axonal protein, giving first clues toward the determination of its functional role. Furthermore, information about the mode of action of the environmental modulation, stimulatory or inhibitory, may be derived. For a definite assignment of the modulated proteins to concomitantly modulated functions, it will be necessary, as a next step, to develop quantitative assays for axonal functions, such as axon elongation, sprouting, filopodial motility or the capacity for synapse formation, in order to use them in joint analyses with the modulation of axonal protein expression during experimental variations of the local environment of axons. Proteins so assigned to axonal functions may then by investigated for further detail by cell biological, biochemical, and recombinant DNA technologies, to obtain insight into the molecular details of the processes acting in the implementation and regulation of axonal functions.

We thank G. Guroff for generously supplying us with nerve growth factor, M. Bokoum and L. Bowers for technical help, R. Billeter for help and advice with gel electrophoresis, and G. Knott and M. Shapiro for advice on statistical procedures. P.S. was supported during the course of this work by fellowships from the Swiss National Science Foundation, the Schweizerische Stiftung fuer medizinisch-biologische Stipendien, the Roche Research Foundation, and the Geigy-Jubilaeums-Stiftung.

REFERENCES

ABNEY, E. R., BARTLETT, P. P., and RAFF, M. C. (1981). Astrocytes, epencymal cells, and oligodendrocytes develop on schedule in dissociated cultures of embryonic rat brain. *Dev. Biol.* **83,** 301–310.

ANDERBERG, M. R. (1973). "Cluster Analysis for Applications" Chap. 6. Academic Press, New York.

ANDERSON, N. L., TAYLOR, J., SCANDORA, A. E., COULTER, B. P., and ANDERSON, N. G. (1981). The TYCHO system for computer analysis of two-dimensional gel electrophoresis patterns. *Clin. Chem.* **27,** 1807–1820.

ANDERSON, L., HOFMANN, J.-P., GEMMELL, A., and TAYLOR, J. (1984). Global approaches to the quantitative analysis of gene-expression patterns observed by two-dimensional gel electrophoresis. *Clin. Chem.* **30,** 2031–2036.

BARNSTABLE, C., JESSELL, T., SANES, J., STEVENS, C., and ROBERTSON, M. (1983). How molecular is neurobiology? *Nature (London)* **306,** 14.

BARON VAN EVERCOOREN, A., KLEINMAN, H. K., OHNO, X., MARANGOS, P. SCHWARTZ, J. P., and DUBOIS-DALQ, M. E. (1982). Nerve growth factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* **8,** 179–183.

CAMPENOT, R. B. (1977). Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **74,** 4516–4519.

CAMPENOT, R. B. (1979). Independent control of the local environment of somas and neurites. *Methods Enzymol.* **58,** 302–307.

EDGAR, D., TIMPL, R., and THOENEN, H. (1984). The heparin-binding domain of laminin is responsible for its effect on neurite outgrowth and neuronal survival. *EMBO J.* **3,** 1463–1468.

FISCHBACH, G. D. and NELSON, P. G. (1976). Cell cultures in neurobiology. *In* "Handbook of Physiology. The Nervous System I," pp. 719–779. Amer. Physiol. Soc., Washington, D.C.

GREENE, L. A. (1984). The importance of both early and delayed responses in the biological actions of nerve growth factor. *Trends NeuroSci.* **7,** 91–94.

GUNDERSEN, R. W., and BARRETT, J. N. (1979). Neuronal chemotaxis: Chick dorsal root axons turn toward high concentrations of nerve growth factor. *Science* **206,** 1079–1080.

HALL, M. E., WILSON, D. L., and STONE, G. C. (1978). Changes in synthesis of specific proteins following axotomy: Detection with two-dimensional gel electrophoresis. *J. Neurobiol.* **9,** 353–366.

HARTIGAN, J. A. (1975). "Clustering Algorithms." Wiley, New York.

LEMKIN, P. F., and LIPKIN, L. E. (1983a). 2D-Electrophoresis gel data base analysis: Aspects of data structures and search strategies in GELLAB. *Electrophoresis* **4,** 71–81.

LEMKIN, P. F., and LIPKIN, L. E. (1983b). Database techniques for two-dimensional electrophoretic gel analysis. *In* "Computing in Biological Science" (M. Geisow and A. Barret, eds.) pp. 181–231. Elsevier, Amsterdam.

LEMKIN, P. F., LIPKIN, L. E., and LESTER, E. P. (1982). Some extensions to the GELLAB two-dimensional electrophoretic gel analysis system. *Clin. Chem.* **28,** 840–849.

LEMKIN, P. F., SONDEREGGER, P., and LIPKIN, L. E. (1984). Identification of coordinate pairs of polypeptides: A technique for screening of putative precursor product pairs in 2D gels. *Clin. Chem.* **30,** 1965–1971.

LETOURNEAU, P. C. (1975). Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* **44,** 92–101.

LEVI-MONTALCINI, R., MEYER, H., and HAMBURGER, V. (1954). *In vitro* experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer Res.* **14,** 49–57.

LEVINE, J., SKENE, P., and WILLARD, M. (1981). GAPs and fodrin: Novel axonally transported proteins. *Trends NeuroSci.* **4,** 273–277.

LIESI, P. (1985). Do neurons in the vertebrate CNS migrate on Laminin? *EMBO J.* **4,** 1163–1170.

LIESI, P., DAHL, D., and VAHERI, A. (1983). Laminin is produced by early rat astrocytes in primary culture. *J. Cell Biol.* **96,** 920–924.

LIPKIN, L. E., and LEMKIN, P. F. (1980). Data-base techniques for multiple two-dimensional polyacrylamide gel electrophoresis analysis. *Clin. Chem.* **26,** 1403–1412.

LORENZ, T., and WILLARD, M. (1978). Subcellular fractionation of intra-axonally transported polypeptides in the rabbit visual system. *Proc. Natl. Acad. Sci. USA* **75,** 505–509.

MOJENA, R. (1975). Hierarchical grouping methods and stopping rules: An evaluation. *Computer J.* **20,** 359–363.

MUDGE, A. W. (1981). Effect of chemical environment on levels of substance P and somatostatin in cultured sensory neurones. *Nature (London)* **292,** 764–767.

MUDGE, A. W. (1984). Schwann cells induce morphological transformation of sensory neurones *in vitro*. *Nature (London)* **309,** 367–369.

NEWMARK, P. (1983). An embarrassment of peptides. *Nature (London)* **303,** 655.

O'FARRELL, P. H. (1975). High resolution two-dimensional electrophoresis. *J. Biol. Chem.* **250,** 4007–4021.

PATTERSON, P. H. (1978). Environmental determination of autonomic neurotransmitter function. *Annu. Rev. Neurosci.* **1,** 1–17.

PERKINS, C. S., CARLSTEDT, T., MIZUNO, K., and AGUAYO, A. J. (1980). Failure of regenerating dorsal root axons to regrow into the spinal cord. *Can. J. Neurol. Sci.* **7,** 323.

PERRY, G. W., and WILSON, E. L. (1981). Protein synthesis and axonal transport during nerve regeneration. *J. Neurochem.* **37,** 1203–1217.

RAMON Y CAJAL, S. (1928). "Degeneration and Regeneration of the Nervous System" (translated by R. M. May), Oxford Univ. Press, London.

REIER, P. J., STENSAAS, L. J., and GUTH, L. (1983). The astrocytic scar as an impediment to regeneration in the central nervous system. *In*

"Spinal Cord Reconstruction" (C. C. Kao, R. P. Bunge, and P. J. Reier, eds.), pp. 163–195. Raven Press, New York.

ROTSHENKER, S. (1981). Sprouting and synapse formation by axons while separated from their cell bodies. *Soc. Neurosci.* Abstr. **11,** 37.

SHAPIRO, M. (1983). "C-LAB Applications Manual," 1st ed. LSM, DCRT, NIH, Bethesda, Md 20205.

SKENE, J. H. P. (1984). Growth-associated proteins and the curious dichotomies of nerve regeneration. *Cell* **37,** 697–700.

SKENE, J. H. P., and WILLARD, M. (1981). Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. *J. Cell Biol.* **89,** 86–95.

SONDEREGGER, P., FISHMAN, M. C., BOKOUM, M., BAUER, H. C., NEALE, E. A., and NELSON, P. G. (1984). A few axonal proteins distinguish ventral spinal cord neurons from dorsal root ganglion neurons. *J. Cell Biol.* **98,** 364–368.

SONDEREGGER, P., FISHMAN, M. C., BOKOUM, M., BAUER, H. C. and NELSON, P. G. (1983). Axonal proteins of presynaptic neurons during synaptognesis. *Science* **221,** 1294–1297.

SONDEREGGER, P., LEMKIN, P. F., LIPKIN, L. E., and NELSON, P. G. (1985). Differential modulation of the expression of axonal proteins by non-neuronal cells of the peripheral and central nervous system. *EMBO J.* **4,** 1395–1401.

SPENCER, B. (1984). Cluster analysis. *Byte* **8,** 129–130 and 423–426.

SPERRY, R. W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc. Nat. Acad. Sci. USA* **50,** 703–710.

STENSAAS, L. J., BURGESS, P. R., and HORCH, K. W. (1979). Regenerating dorsal root axons are blocked by spinal cord astrocytes. *Soc. Neurosci.* **5.** 684 (Abstract).

SUTCLIFFE, J. G., and MILNER, R. J. (1984). Brain specific gene expression. *Trends NeuroSci.* **7,** 95–99.

WILSON, D. L., and STONE, G. C. (1979). Axoplasmic transport of proteins. *Annu. Rev. Biophys. Bioeng.* **8,** 27–45.