Differential modulation of the expression of axonal proteins by non-neuronal cells of the peripheral and central nervous system

P. Sonderegger1,2, P. F. Lemkin3, L. E. Lipkin3 and P. G. Nelson1

1Laboratory of Developmental Neurobiology, National Institutes of Child Health and Human Development, National Institutes of Health, Bldg 36, Rm 2A/21, Bethesda, MD 20205, USA. 2Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, and 3Image Processing Section, Laboratory of Mathematical Biology, National Cancer Institute, Building 36, Room 417, Bethesda, MD 20205, USA

Communicated by M. Cuenod

Axonal behavior during the formation of the neuronal network of the nervous system has been shown to be under environmental control. Hence, as a first step in a project aiming to elucidate the molecular basis of axonal functions, we have identified axonal proteins whose synthesis is subject to environmentally induced changes. Neurons from chicken embryonic dorsal root ganglia (DRG) were grown in a compartmental cell culture system that allows selective examination of axonal proteins. Non-neuronal cells of the peripheral or central nervous system were co-cultured with the DRG axons. The axonal proteins expressed under these different environmental conditions were examined by metabolic labeling and two-dimensional SDS-polyacrylamide gel electrophoresis. Computerized quantification revealed that 12 out of 400 axonal proteins responded to changes in the local axonal environment by a change in their relative abundance. Some proteins changed in response to both types of co-cultures whereas some changed specifically under the influence of either peripheral or central non-neuronal cells.

Key words: axon development/axonal proteins/dorsal root ganglia/electrophoresis/image processing

Introduction

The capability of neurons to extend their axons over long distances along a specified pathway to make specific contact with target neurons is one of the crucial features in the topographic organization of the nervous system. The axonal functions that implement this network formation are phenomenologically described as elongation, sprouting, pathfinding, fasciculation, synthesis of a specific neuro-transmitter and synapse formation. However, the molecular building blocks underlying axonal functions are only partially identified, and even less is known about their mechanism of action. In development as well as in regeneration, the expression of axonal functions is epigenetically influenced by the axons' cellular and humoral environment (Ramon y Cajal, 1928; Levi-Montalcini et al., 1954; Sperry, 1963; Patterson, 1978; Letourneau, 1982). As for sensory neurons, environmental influences have been reported to act on axon elongation (Levi-Montalcini et al., 1954; Richardson and Ebenhald, 1982; Baron van Evercooren et al., 1982), the guidance of the axonal pathway (Letourneau, 1975; Gundersen and Barrett, 1979), the choice of the neurotransmitter (Mudge, 1981) and the morphology of the axons' initial segment (Mudge, 1984). Studies after injury of dorsal roots have revealed that regenerating dorsal root ganglia axons virtually stop elongating at the transition from the peripheral to the central nervous system (Perkins, et al., 1980; Stensaas et al., 1979; Reier et al., 1983). Conversely, a peripheral nerve segment that was grafted into mammalian spinal cord was able to support re-outgrowth of axons from central neurons markedly better than the central glial cells normally present (Richardson et al., 1980; Benfey and Aguayo, 1982). These findings corroborate Ramon y Cajal's hypothesis that peripheral glia are more favorable to axon elongation than central glia (Ramon y Cajal, 1928). The virtual absence of structural and functional repair in the central nervous system is thought to depend on the impeding action of the central glia on the re-outgrowing axons. However, it is still an unsettled issue whether the adverse action of central glia is due to as mechanical barrier of scar tissue or to environmentally induced physiological changes in the intrinsic capability of neurons to regenerate their axons (Reier et al., 1983).

As a first step in a project aiming towards the elucidation of the molecular basis of axonal functions and their environmental regulation, we examined whether the protein composition of axons was subject to environmentally induced changes. We have previously reported on a compartmental cell culture system allowing study of the expression of axonally transported proteins in axons that are embedded in a complex and variable local cellular environment (Sonderegger et al., 1983, 1984). In the present study dorsal root ganglia (DRG) cells from 10-day-old chicken embryos were cultured in a compartmental cell culture system, (Campenot, 1977, 1979) that allows separate access to neuronal cell somas and to their axons. Three days after the dissociated DRG cells were plated in the central compartment, the first axons had grown across the barrier between the compartments and appeared in the side compartments. The neuronal cell bodies, however, were retained in the central compartment. After 10 days in culture, the neurons and accompanying non-neuronal cells of the central compartment had re-associated in a histotypic fashion (Sonderegger et al., 1983, 1984), whereas the axons in the side compartments had extended and associated into thick fascicles (Figure 2a). Both peripheral non-neuronal cells, obtained from DRGs, and central non-neuronal cells, obtained from spinal cord (SC), were co-cultured with the axons (Figure 2b and c, respectively). 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 [35S]methionine, added to the neuronal somas in the central compartment. After incubation for 40 h, the cellular material of the side (axonal) compartments was harvested. This system thus ensures that the proteins synthesized in the neuronal somas of the central compartments and incorporated into the axons extending into the side compartments are the only radioactively labeled proteins to be found in the side compartments (Sonderegger et al., 1983). The side compartment proteins were then subjected to two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) followed by fluorography (Bon-
ner and Laskey, 1974). Axonal proteins that were synthesized and axonally transported when the axons were in contact with either peripheral (DRGNN) or central (SCNN) non-neuronal cells were quantified and compared with the proteins that were synthesized when the axons were grown without any co-cultured cells, using GELLAB, a system for computerized image analysis (Lipkin and Lemkin, 1980; Lemkin et al., 1982; Lemkin and Lipkin, 1983a, 1983b, 1983c).

Results

Composition of the cells used for co-culture

The cells used for co-culture with the DRG axons were obtained from dorsal root ganglia and spinal cord, respectively. To obtain neuron-free DRG cultures, the somas of the DRG neurons were mechanically removed from cultures of non-dissociated ganglia. To obtain neuron-free SC cultures, the spinal cords were dissected at day 14, a time previously found unfavourable for the survival of neurons in culture, and kept for 3 days in fetal calf serum, a condition found adverse to the generation of large unequivocally identifiable spinal cord neurons as determined by morphological and electrophysiological criteria. The cellular composition of these cultures was assessed using cell type-specific markers (for review, cf. Raff et al., 1979; Roots, 1981; Schachner, 1982). As a marker for astrocytes, the presence of glial fibrillary acidic protein (GFAP) was assessed (Bignami and Dahl, 1977). The cell surface antigens 01 and 04 were used to identify mature and immature oligodendrocytes, respectively (Sommer and Schachner, 1981; Schachner et al., 1981). A2B5, a cell surface antigen initially found on chicken retina (Eisenbarth et al., 1979), was used in combination with GFAP and 01/04 to identify neurons and to estimate the proportion of immature cells among astrocytes and oligodendrocytes (Schnitzer and Schachner, 1982; Berg and Schachner, 1982). S-100 protein (Cicero et al., 1970; Ghandour et al., 1981) served as marker for Schwann cells in the DRG-derived cultures and the presence of fibronectin was taken as a tag of fibroblasts (Schachner et al., 1978; Paetau et al., 1980).

Dissection and media conditions were identical to those used in the metabolic labeling studies. Immunofluorescent stainings were scheduled for a time equivalent to the middle of the metabolic labeling period; hence, stainings were done on ‘secondary cells’ at day four after replating.

Among the SC cells, ~60% were GFAP⁺, prevalently fibroblastic in morphology. Process-bearing cells, which attracted attention by distinctly stronger staining, represented ~5% of the GFAP⁺ cells. Occasionally, packed patches of process-bearing GFAP⁺ cells could be observed (Figure 1b). A2B5, a cell surface marker previously found to stain neurons (Eisenbarth et al., 1979) and glia cells (Schnitzer and Schachner, 1982; Raff et al., 1983), was present on ~40% of the cells (Figure 1c). Among
the fibroblastic GFAP+ cells, ~40% were A2B5+, as revealed by double staining. This high percentage of A2B5+ cells may indicate that a substantial proportion of the fibroblastic astrocytes were immature (Schnitzer and Schachner, 1982; Berg and Schachner, 1982). From the process-bearing GFAP+ cells, an estimated 10% were A2B5+. About 2–3% of the cells were 01+ and 15% were 04+. The majority of the 01+ cells resembled process-bearing oligodendrocytes as described previously in murine cultures (Sommer and Schachner, 1981) and, in double stainings ~30% of them were A2B5+. The 04+ cells were prevalently of fibroblastic morphology (Figure 1d) and ~80% were also A2B5+. This marker distribution indicates that most oligodendrocytes were immature (Sommer and Schachner, 1981; Schachner et al., 1981).

In DRG or SC cultures designed for maximum survival of neurons (Fischbach and Nelson, 1977; Sonderegger et al., 1983), neurons can be identified by morphological criteria and subsequently recorded by physiological techniques. The A2B5 staining pattern of cells considered neuronal by such operational definition, was strikingly different from the staining pattern of fibroblastic A2B5+ cells. Cell somas of chicken spinal cord and dorsal root ganglia neurons showed smooth, diffuse cell surface staining which usually was rather faint, whereas fibroblastic cells were stained in a rough-granular or dot-like manner. Hence, in the absence of an unequivocal marker for neurons, we defined neurons operationally as A2B5+ cells with smooth, regular staining of the cell soma and long, bright processes, that were negative for GFAP and 01 in double stainings with A2B5. In our cultures, only occasional cells fitting this description were found. Although we are not in the position to deliver a strict proof for the complete absence of neurons from these cultures, we consider the conclusion warranted that they were virtually devoid of neurons. Staining for fibronectin revealed the presence of ~20% fibroblasts in low density cultures. Hence, the SC-derived cultures were composed of ~80% glial cells and ~20% fibroblasts. The glial cells were prevalently astrocytic. A considerable proportion of the astrocytes and the major proportion of the oligodendrocytic cells were immature.

The cells from explanted DRGs that were left after the neuronal cell somas were excised, were ~70–80% S-100+ (Figure 1a). The remaining cells were fibronectin+. Only occasional A2B5+ and GFAP+ and 04+ cells were found. The 04+ cells, when present, occurred in clusters of up to 10 cells and were A2B5−. No 01+ cells were observed. Hence, the DRG-derived cells used for co-culture were mainly composed of Schwann cells and contained a smaller fraction of fibroblasts.

**The pattern of fasciculation of DRG axons is specifically modulated by co-cultured non-neuronal cells**

Light microscopic inspection of the cell cultures of all three experimental conditions did not reveal any sign of deteriorating neurons or axons until the end of the experiments. However, co-culture with central non-neuronal cells (Figure 2c) led to a distinctly finer branching pattern of the axons with considerably less extensive fasciculation than the axons that were without co-cultured cells (Figure 2a) or those co-cultured with peripheral non-neuronal cells (Figure 2b). The degree of fasciculation has been found to depend on the strength of the adhesive forces acting among axons relative to the adhesion between axons and the environmental surfaces (Rutishauser et al., 1978). The observed fasciculation pattern thus suggests that the adhesive forces occurring under co-culture with central non-neuronal cells tend more towards adhesion with the surface than under the conditions where DRG axons are grown without any co-cultured cells or when peripheral non-neuronal cells are co-cultured. This difference in fasciculation may be due to higher adhesive properties of central non-neuronal cells or a subset thereof with respect to peripheral non-neuronal cells or collagenized cell culture plates. Alternatively co-culture with central non-neuronal cells may alter the adhesive properties on the axonal surface resulting in lower affinity among axons or higher affinity of axons to non-neuronal cells.

**The expression of a few proteins is modulated by co-culture with peripheral and central non-neuronal cells**

The axonal proteins that were newly synthesized and transported to the axons during the period of labeling were analyzed by two-dimensional SDS-PAGE (O’Farrell, 1975). Under the conditions employed for these experiments, > 400 protein spots were discernible on each gel. Computerized quantitation of the individual protein spots using GELLAB (Lemkin and Lipkin, 1983b) revealed that the bulk of the axonal proteins were expressed at similar relative quantities under all three experimental conditions. This finding was not surprising, since the constancy of the gross morphology (with the exception of the fasciculation pattern) caused us to expect constancy of the bulk of the structural proteins, which comprise a substantial proportion of the major cellular proteins.

Twelve proteins, out of a total of > 400 discernible spots, were
of the absolute DRG. We modulatory The data indicate that most of the axonal proteins might possibly be generated by mitotic expansion of a subset of neurons or by differential survival of neuronal or axonal subpopulations. However, the DRGs were dissected after the DRG neurons had become post-mitotic during embryonic development (McMillan Carr and Simpson, 1978) and DRG neurons of 10-day-old chicken embryos stay post-mitotic after being brought into tissue culture (Chalazonitis and Fischbach, 1980). The compartmental cell culture system employed (Sonderegger et al., 1983, 1984) allows manipulation of the local, humoral or cellular environment of the axons while the milieu of the neuronal somas is kept constant. In the present series of experiments the neuronal somas were exposed in all experimental classes, at all times, to a constant environment of peripheral non-neuronal cells — and never to central non-neuronal cells. The axons, on the other side, were exposed to another cellular environment in each experimental class. Hence, this system imitates the in vivo situation closer than do the usual dissociated cell cultures, while still providing the advantages of the in vitro approach.

The non-neuronal cells to be co-cultured were added locally to the axons extended to the side compartments at a point in time when the axons spanned more than 2/3 of the width of the side compartment (day 7). At this time, the DRG cells of the central compartment (neurons + non-neuronal cells) had resumed a pattern histotypic for DRGs. No morphological signs for neuronal cell death were observed in the central compartments and no morphological signs for axonal deterioration were detected, till the end of the experiments, that could cause selective survival of axons of one or the other axonal subpopulation.

This experimental paradigm and the light microscopic observations made during the course of the experiments thus substantiate the assumption that the same neurons were present in all experiments and throughout all experimental classes. With respect to the axons in the side compartments, what we can tell with certainty is that the same axons were present in all experimental conditions at the time when the non-neuronal cells were added for local co-culture, that no substantial degradation of axons occurred during the time the axons were exposed to co-cultured cells and that the amount of radioactivity incorporated into the axonal proteins under all three experimental conditions was in the same range. Thus, we consider the observed phenomenon most probably to be due to modulatory effects on the protein synthesis of a pre-existing set of cells and their axons. We cannot substantiate whether the different local axonal environments affected protein expression and particular axonal functions, such as growth, uniformly in all axons present. To address these questions experimentally is something for future studies.

Our observations show that the local cellular environment of the axons specifically influences their protein composition. Thus, local signals impinging on neuronal axons must be capable of regulating biosynthetic activity or translocation mechanisms or both in the neuronal cell body or to activate local post-translational modifications in the axons. The system used in the present study allows the analysis of this process which may be of unique importance in the development of the nervous system. Modification in the protein content of axons may represent the macromolecular correlate of modulation in any, yet undetermined, axonal function such as elongation, sprouting, fasciculation, transmitter synthesis or the capability for synapse formation. The difference in

### Table 1

<table>
<thead>
<tr>
<th>Subset</th>
<th>Spot no.</th>
<th>Co-cultures compared (Ratio of mean normalized densities)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRGNN versus none</td>
<td>SCNN versus none</td>
<td>SCNN versus DRGNN</td>
</tr>
<tr>
<td>I</td>
<td>435</td>
<td>0.51&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>582</td>
<td>0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>335</td>
<td>0.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>516</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>86</td>
<td>3.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>85</td>
<td>(0.59)</td>
</tr>
<tr>
<td>II</td>
<td>90</td>
<td>(1.76)</td>
</tr>
<tr>
<td>II</td>
<td>484</td>
<td>(1.30)</td>
</tr>
<tr>
<td>III</td>
<td>162</td>
<td>1.84&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>359</td>
<td>(1.79)</td>
</tr>
<tr>
<td>IV</td>
<td>361</td>
<td>(0.82)</td>
</tr>
<tr>
<td>IV</td>
<td>462</td>
<td>(1.14)</td>
</tr>
</tbody>
</table>

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 three experimental classes of co-culture, namely DRG axons without co-cultured cells (none), DRG axons with co-cultured peripheral non-neuronal cells (DRGNN) and DRG axons with co-cultured central non-neuronal cells (SCNN). If a statistically significant difference of the normalized density values for a protein spot occurred between two experimental classes (F-test, n=5) at a confidence level of 0.95, the ratio value is marked by (a). If a difference was revealed at a confidence level of 0.90, the ratio value is labelled by (b). If no difference was revealed by the statistical test, the ratio of the mean normalized densities of the compared experimental classes is in parentheses. The environmentally modulated proteins were subdivided into four subsets, according to the pattern of differences observed in the comparison of the three experimental classes: proteins changed by both peripheral and central non-neuronal cells to the same extent (subset I protein); proteins changed by peripheral and central non-neuronal cells to a different extent and possibly in opposite directions (subset II); proteins changed specifically by peripheral non-neuronal cells (subset III) and proteins changed specifically by central non-neuronal cells (subset IV); subset numbers in parentheses indicate proteins that could be assigned only tentatively to a particular subset because of absence of complementarity in the statistical comparisons between the three experimental classes.

found to differ in at least one of the three possible comparisons (Table I: DRGNN versus none, SCNN versus none, SCNN versus DRGNN). These proteins were subdivided into four subsets according to the pattern of differences they showed (Table I and Figure 3). Proteins 435 and 582, and possibly 335 and 516, were changed under the influence of both peripheral and central non-neuronal cells to about the same extent (Table I: subset I proteins). Protein 86, and possibly 85, 90 and 484, were changed by both peripheral and central non-neuronal cells, but the extent of the induced changes differed (subset II). Protein spot 162, and possibly 359, were changed specifically by co-culture with peripheral, but not with central, non-neuronal cells (subset III), whereas proteins 462 and 361 were specifically changed under the influence of central, but not peripheral non-neuronal cells (subset VI).

### Discussion

The data presented indicate that non-neuronal cells from the peripheral and the central nervous system exert different modulatory influences on the expression of axonal proteins in DRG. We point out that these data do not represent comparisons of absolute amounts of axonal proteins but rather comparisons of the relative amounts of proteins that were produced and axonally transported during a 40 h period between the third and fifth day after start of the co-culture. The ratios of the absolute amounts of a particular protein would be expected to be a complex function of synthesis, axonal transport, post-translational modification and degradation. Differences in the pattern of axonal proteins might possibly be generated by mitotic expansion of a subset of neurons or by differential survival of neuronal or axonal subpopulations.
the fasciculation patterns observed when axons were embedded in peripheral and central non-neuronal cells (Figure 2) may indeed serve as an illustration of such an environmentally induced behavioural change. The changes in the synthesis of axonal proteins of DRG neurons induced by the local environment of the axons include changes evoked specifically by either peripheral or central non-neuronal cells and changes that are induced by the presence of both peripheral and central non-neuronal cells. The cell types responsible for the registered changes remain to be determined, since both the peripheral and the central non-neuronal cell populations used for co-culture with the axons are heterogeneous (Figure 1). Future experiments will aim to determine the modulatory effects of enriched glial subpopulations onto the expression of axonal proteins and to correlate environmentally induced changes in the synthesis of axonal proteins with changes in particular axonal functions. This should contribute to the molecular understanding of axonal features that have wide biological and medical implications during developmental and regenerative periods of the nervous system.

Materials and methods

Three-compartment cell culture system

The three-compartment cell culture system was set up as described in detail by its designer (Campenot, 1977, 1978). To give the outgrowing axons direction ~15 parallel scratches, ~0.5 mm apart, were made across the surface of a collagenized, dry, 35-mm cell culture dish (Falcon Labware, Oxnard, CA). A drop of ~100 μl of a film-forming medium composed of 0.6% hydroxypropyl methylcellulose (Methocel E4M Premium, Dow Chemical Co., Indianapolis, IN) was deposited in the middle of the scratches. The Teflon inset, covered on its bottom side with silicon vacuum grease (Dow Chemical Co.), was then placed into the dish so that the scratches spanned all three compartments. The system was tested for absence of hydrostatic bulk flow between the compartments by filling the two side compartments with 0.5 ml of growth medium whereas the central compartment was left empty. Only those plates that had no leakage of medium into the central compartment during 4–6 h were used.

Cell cultures

The cell culture growth medium used for all conditions was composed of Eagle's minimal essential medium in Earle's salt solution (Gibco), 5% heat-inactivated horse serum (Gibco), 2.5% chicken embryo extract, nerve growth factor (25 ng/ml) and nutrient mixture containing transferrin (200 μg/ml), insulin (10 μg/ml), triiodothyronine (20 ng/ml), progesterone (40 nM), corticosterone (0.2 μg/ml), putrescine (200 μM) and sodium selenite (60 nM). Dorsal root ganglia were dissected from 10-day-old chicken embryos, cleaned of adherent connective tissue, and incubated in 0.25% trypsin (Gibco Laboratories) and 0.02% DNase I (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C for 30 min. The ganglia were dissociated to single cells by trituration and counted in a modified Fuchs-Rosenthal chamber. Between 60 000 and 90 000 cells were plated in the central compartment. After 3 days, the first axons, accompanied by some non-neuronal cells appeared in the two side compartments. However, the film of medium under the barrier between the central compartment and the side compartment was thin enough to prevent passage of neuronal cell bodies. Excessive multiplication of rapidly dividing non-neuronal cells that had migrated from the central compartment into the side compartment during the first days in culture was inhibited by...
supplementing the side compartment medium with 0.12 mM 5-fluorodeoxyuridine (Sigma Chemical Co., St. Louis, MO)/0.3 mM tritium (Sigma Chemical Co.) for 24 h starting at day 4. After ~1 week in culture, the axons in the side compartments had grown together into thick fascicles.

Peripheral non-neuronal cells from DRG were obtained essentially as described previously (Wood, 1976) by growing non-dissociated DRGs in collagen-covered dishes in the presence of 25 ng/ml nerve growth factor; however, no anti-muscle serum was used. One week later, the outgrowing axons were surrounded by a dense layer of non-neuronal cells that had migrated out of the ganglion, whereas the neuronal cell somas were still aggregated. To obtain a neuron-free population of non-neuronal cells, the aggregates of neuronal somas were cut out. The non-neuronal cells left behind were dissociated with trypsin and EDTA, counted and added to the axons in the side compartments (~1 000 cells per compartment).

Central non-neuronal cells were obtained by cultivating dissociated spinal cord cells from 14-day-old chicken embryos for ~1 week in the same medium that was used for the neuronal and the peripheral non-neuronal cells, except that for the first 3 days, the horse serum was replaced by fetal bovine serum. For co-culture with DRG axons, the cells were dissociated with trypsin and EDTA as described for the peripheral glial cells, counted and plated into the side compartment.

Selective metabolic labeling of axonal proteins
The newly synthesized proteins were labeled by addition to the central compartment of labeling medium composed of methionine-free growth medium substituted with 15 µM unlabeled methionine, and 1 µCi/ml [%55]methionine (~1000 Ci/mmol, New England Nuclear, Boston, MA). The side compartments contained the same medium, except that 4 mM unlabeled methionine replaced the radioactive methionine. Typically, material from 2–3 plates was needed for one plate of side gel. Incubations of 4 h were used to allow for accumulation of the proteins of the peripheral transport reticles (Wisen & Stone, 1979). After labeling, 50 µl of medium from each compartment was aspirated. The protein was precipitated by trichloroacetic acid (TCA), and the free [%55]methionine that remained in solution was counted in a β-scintillation counter at a counting efficiency of ~70%. This procedure served to provide an estimate of the leakage of radioactive label into the side compartment. After the remainder of the supernatant medium had been removed, 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 gel electrophoresis
Two-dimensional SDS-PAGE was done essentially as developed by O’Farrell (1975). Samples were matched for TCA-precipitable radioactivity (~400 000 c.p.m./gel). The amphoteric solution of the isoelectric focusing step was composed of 1.6% ampholine 5/7 (LKB) and 0.4% ampholine 3/10. The second dimension was composed of 10% polyacrylamide (BDH) added to prevent cracking of the gels during drying. The preparation of the gels for fluorography was done according to the principles developed by Bonner and Laskey (1974); however, the commercially available acetic acid based enhancer (Enchance, NEN) was used. Fluorographic exposure was done with XOMAT XAR-2 film (Kodak) for 4 weeks at ~70°C.

Quantitative analysis of two-dimensional SDS-PAGE with GELLAB
The GELLAB system for the computerized analysis of two-dimensional SDS-PAGE has been used to find protein differences between different biological states of cells and body fluids (Lipkin and Lemkin, 1980; Lemkin et al., 1982; Lemkin and Lipkin, 1983a, 1983b, 1983c). The fluorographic replicas of the two-dimensional electropherograms are scanned, along with a neutral density calibration wedge, by a high resolution black and white TV camera and digitized into 512 x 512 pixel element images with 256 gray values (white to black). The images are then calibrated in terms of optical density units and thereafter all measurements are in terms of integrated optical density. GELLAB successively segments individual spots in a gel image, pairs corresponding spots from different gels and finally merges corresponding spots into a so-called gel data base. The user selects a referent gel (denoted the Rgel) and interactively defines a small number of corresponding landmark spots between each of the remaining N-I gels and the Rgel. Spots in the N gels which correspond to the same spot in the Rgel are combined in Rspot sets. The data were normalized by the least squares method (Lemkin and Lipkin, 1983c). The N gels were subdivided into subsets of gels which included 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. GELLAB has facilities for interactively searching the composite spot data base as well as saving the results of searches and visualizing them with Rmap and mosaic images. An Rmap is a copy of any of the original gel images with selected Rspots labeled. A mosaic is an image composed of panels of subregions of all of the gel images surrounding a particular Rspot ordered by increasing spot density.

Sources of antibodies
Antiserum against glial fibrillary acidic protein (anti-GFAP) was a gift from R.M. Prus (NIH, Bethesda, MD, USA) and was used at a dilution of 1:1000. Monoclonal antibodies to the glial fibrillary proteins (anti-01 and anti-04, respectively) were provided by M.Schachner (University of Heidelberg, Heidelberg, FRG) and used at 1:100 as described previously (Sommer and Schachner, 1981). Monoclonal antibodies to the cell surface antigen A2B5 (Eisenbarth et al., 1979; Schnitzer and Schachner, 1982) was a gift from M.Nirenberg (NIH, Bethesda, MD, USA) and was used at a dilution of 1:2. Antibodies against various system-specific antigens (anti-S-100 (anti-S-100 and anti-0.95) and anti-fibronectin (anti-fibronectin) were purchased from Dakopatts (Glostrup, Denmark) and used at dilutions of 1:20. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC) was from Cappel (Malvern, PA, USA) and used at 1:100. Fluorescein isothiocyanate-conjugated anti-rabbit IgG was from Sigma (St. Louis, MO, USA) and used at 1:200. Tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (TRITC) was from Nordic (Tilburg, Netherlands) and used at 1:50. Tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (GM-TRITC) was from Zymed (South San Francisco, CA, USA) and was used at 1:100. All antibodies were diluted with PBS containing 10% horse serum (Amired, Basel, Switzerland) and 0.3% gelatin (Sigma, St. Louis, MO, USA).

Indirect immunofluorescent labeling
Marker assays were done on cultured cells from dorsal root ganglia and the spinal cord that have been grown under the same conditions as those used for co-culture and schedule so that the cells’ age in culture corresponded to the middle of the time of the metabolic labeling in the co-cultures, namely four days after trypsinization and replating. Binding of antibodies to the cells surface markers A2B5, 01 and 04 and the extracellular matrix marker fibronectin were done on viable cells at 21°C for 30 min. The cells were washed five times with phosphate buffered saline (PBS) containing 10% horse serum and 0.3% gelatin. The second, fluoresently labeled antibodies were added for an incubation of 45 min at 21°C. After five washes, the cells were fixed in 4% formaldehyde for 30 min at 4°C, washed twice in PBS, immersed in phosphate buffered glycerol and stored at ~20°C. Examination was done with a Leitz microscope equipped with epi-fluorescence illumination. For the study of the intracellular markers GFAP and S-100, the cells were made permeable by treatment with acetic ethanol (95% ethanol/5% acetic acid) for 10 min at ~20°C and subsequently brought back to PBS that was consecutive washed with 90%, 50% and 50% ethanol. Prior to the addition of the first antibodies, the cells were incubated in PBS/10% horse serum/0.3% gelatin at 21°C for 10 min. Tertiary fibroblasts from 11-day-old chicken embryos were co-processed with every staining. None of the markers we used was found present on fibroblasts.

Double staining of A2B5 and GFAP was done by first staining native cells for A2B5, however, for the staining of GFAP fixation was omitted. Subsequently the cells were permeabulated and stained for GFAP. Double staining for A2B5 and 01 or 04 was done by first staining for A2B5 on native cells. Subsequently, without fixation with formaldehyde, the plates were stained for 01 or 04 as indicated above. For quantification in double stainings, at least 100 cells were counted.

Acknowledgements
We thank G.Guroff for supplying us with nerve growth factor, R.M. Prus, M. Schachner and M. Nirenberg for antibodies, M. Bokoum, L. Bowers, M.Lutz and U.Walti for technical help, M. Schwab and M. Chiquet for technical advice on immunostaining procedures, and P. Christen for reading the manuscript. P. Sonderegger was supported during the course of this work by fellowships from the Swiss National Science Foundation and the Schweizerische Stiftung für Medizinisch-Biologische Studien.

References

1400
Environmental modulation of axonal proteins


Received on 3 October 1984; revised on 25 March 1985