

A Few Axonal Proteins Distinguish Ventral Spinal Cord Neurons from Dorsal Root Ganglion Neurons

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ABSTRACT A series of proteins putatively involved in the generation of axonal diversity was identified. Neurons from ventral spinal cord and dorsal root ganglia were grown in a compartmented cell-culture system which offers separate access to cell somas and axons. The proteins synthesized in the neuronal cell somas and subsequently transported into the axons were selectively analyzed by 2-dimensional gel electrophoresis. The patterns of axonal proteins were substantially less complex than those derived from the proteins of neuronal cell bodies. The structural and functional similarity of axons from different neurons was reflected in a high degree of similarity of the gel pattern of the axonal proteins from sensory ganglia and spinal cord neurons. Each axonal type, however, had several proteins that were markedly less abundant or absent in the other. These neuron-population enriched proteins may be involved in the implementation of neuronal diversity. One of the proteins enriched in dorsal root ganglia axons had previously been found to be expressed with decreased abundance when dorsal root ganglia axons were co-cultured with ventral spinal cord cells under conditions in which synapse formation occurs (P. Sonderegger, M. C. Fishman, M. Bokoum, H. C. Bauer, and P. G. Nelson, 1983, *Science [Wash. DC]*, 221:1294–1297). This protein may be a candidate for a role in growth cone functions, specific for neuronal subsets, such as pathfinding and selective axon fasciculation or the initiation of specific synapses. The methodology presented is thus capable of demonstrating patterns of protein synthesis that distinguish different neuronal subsets. The accessibility of these proteins for structural and functional studies may contribute to the elucidation of neuron-specific functions at the molecular level.

The specific macromolecular content of each neuron or group of neurons may specify its behavior in terms of axonal pathfinding and fasciculation and synapse formation (1, 2), and thus direct the topographic arrangement and the formation of specifically targeted connections of cells within the nervous system. The distinctive identity of individual classes of neurons has been well defined electrophysiologically, morphologically, and with respect to presumptive neurotransmitters, but less so with regard to their distinctive molecular composition. The probes for macromolecular differences between nerve cells include the binding of antibodies (3–6) or lectins (7–10), and the detection of transmitter-related enzymes (11–13). In recent studies, cellular and extracellular proteins expressed with different abundance in cultured sympathetic neurons expressing adrenergic or cholinergic phenotypes have been identified (14, 15). To relate proteins to developmental axonal

functions, such as pathfinding and synapse formation, we thought it important to perform a systematic search for the axonal proteins expressed in some subsets of the neurons but not in others.

The compartmental cell-culture system devised by Campenot (16, 17) allows the axons of cultured neurons to grow out beneath a thin film of medium into the side compartments of the system, whereas the cell somas are retained in the center compartment. This system allows separate access to axons and cell bodies. The application of a radioactive amino acid to the cell bodies in the center compartment leads to radioactive labeling of newly synthesized neuronal proteins. After a short time, axonal proteins reach their final location by axonal transport. Thus, they can be investigated separately by collection of axonal material from the side compartments. We have previously reported the use of this system for the

study of changes in the axonal protein composition that occur when dorsal root ganglia (DRG)¹ axons were co-cultured with ventral spinal cord (VSC) cells under conditions that lead to synapse formation (18). In the present paper we report differences in the composition of axonal proteins of two distinct neuronal populations, namely those from the dorsal root ganglia and those from the ventral horn of the spinal cord.

MATERIALS AND METHODS

Three-compartment Cell-Culture System: The three-compartment cell-culture system was set up as described in detail by Campenot (16, 17). To give the outgrowing axons direction, about 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 μ l of a film-forming medium composed of 0.6% hydroxypropyl methylcellulose (Methocel E4M premium, Dow Chemical Co., Indianapolis, IN) in F12 medium (Gibco Laboratories, Grand Island, NY) was deposited at the middle of the scratches, and the Teflon inset, covered on its bottom side with silicon high vacuum grease (Dow Chemical Co.), was placed into the dish so that the scratches spanned all three compartments (Fig. 1a). 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 center compartment was left empty. Only those plates that had no leakage of medium into the center compartment during 4 to 6 h were used.

Cell Cultures: Dorsal root ganglia were dissected from 10-d-old chicken embryos, cleaned of adherent connective tissue, and incubated in 0.25% trypsin (Gibco Laboratories) and 0.02% desoxyribonuclease I (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C for 30 min. The digestion solution was removed, and DRG growth medium composed of Eagle's minimal essential medium (MEM) in Earle's salt solution (Gibco Laboratories), 10% heat-inactivated horse serum (Gibco Laboratories), 5% chicken embryo extract, and 25 ng/ml nerve growth factor (kindly provided by G. Guroff, National Institutes of Health) was added. 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 center compartment. After 3 d, 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 center compartment and the side compartments was thin enough to prevent passage of neuronal cell bodies (Fig. 1, b and d). Excessive multiplication of rapidly dividing non-neuronal cells that had migrated from the center 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 uridine (Sigma Chemical Co.) for 24 h starting at day 4. After ~1 wk in culture the axons in the side compartments had grown together into thick fascicles (Fig. 1, c and e).

Spinal cords were removed from 6-d-old chick embryos and, to enrich for motoneurons, the ventral horn was dissected (19). Tryptic dissociation was done as described for DRG. The growth medium for the VSC was composed of MEM, 10% heat-inactivated horse serum, 5% chicken embryo extract, and was conditioned for 1 d with cultured chick myotubes for enrichment by axon growth-promoting factor (20). Approximately 100,000 VSC cells were plated in the center compartment. Extension of VSC axons to the side compartments was slightly slower than that of DRG axons (first appearance of VSC axons in side compartment occurred at ~3.5 d in culture). The multiplication of non-neuronal cells was controlled as described for the DRG cultures.

Selective Metabolic Labeling of Axonal Proteins: The newly synthesized proteins were labeled by addition to the center compartment of labeling medium composed of methionine-free MEM, 10% heat-inactivated horse serum, 5% chicken embryo extract, 25 ng/ml nerve growth factor, 15 μ M unlabeled methionine, and 1 mCi/ml [³⁵S]methionine (~1,000 Ci/mMol, New England Nuclear, Boston, MA). The side compartments contained the same medium, except that 4 mM unlabeled methionine was substituted for radioactive methionine. Typically, material from three to five plates was needed for one polyacrylamide gel. Incubations of 40 h were used to allow for accumulation of the proteins of all axonal transport rate classes (21). After labeling, 50 μ l of medium from each compartment was aspirated, the protein was precipitated by trichloroacetic acid, and the free [³⁵S]methionine that remained in solution was counted on a beta 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 2-dimensional electrophoresis.

Radioautography of Cells Grown in the Compartmental Cell Culture System: Standard compartmented cell cultures were labeled by addition of [³⁵S]methionine to the center compartment under the conditions described previously. After incubation for 40 h, all compartments were washed six times with MEM in the presence of the Teflon inset and fixed with 2.5% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4 for 15 min. The fixative solution was replaced with MEM, and the Teflon inset was carefully removed. After three washes with MEM, fixation with glutaraldehyde was continued for 20 min. The cultures were then rinsed overnight in 0.15 M sodium cacodylate, pH 7.4 and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate. For radioautography, the cultures were coated with NTB3 nuclear track emulsion diluted 2:1 with water at 42°C, dried for 2 h, and exposed at 4°C for 24 h. Radioautograms were developed in Dektol, diluted 1:1, at 16°C for 3 min, fixed for 6 min, and photographed with phase contrast optics.

Two-dimensional Gel Electrophoresis: Two-dimensional SDS PAGE was done essentially as developed by O'Farrell (22). Samples were matched for trichloroacetic acid-precipitable radioactivity (~400,000 cpm/gel). The ampholine 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 run in a 10–17.5% acrylamide gradient with 0.3% linearly polymerized 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 (23); however, the commercially available, acetic acid-based enhancer (EN³HANCE, NEN) was used. Fluorographic exposure was done with XOMat XAR-2 film for 4–5 wk at -70°C.

RESULTS AND DISCUSSION

The compartmental cell culture system allowed the axons of DRG neurons and VSC neurons, respectively, to grow out beneath a thin film of medium into the side compartments of the system, whereas the cell somas were retained in the center compartments. The separate accessibility to neuronal cell somas and axons was exploited to obtain a pure representation of axonally transported proteins.

The compartments of this multicompartment cell culture system were connected to each other by a thin film of medium. It is critical for the success of these experiments that only those plates without bulk flow between the compartments are used for experiments. However, even in plates where no bulk flow of medium between the compartments occurred, contamination of the axonal proteins might result from the migration of heavily labeled non-neuronal cells from the center compartment to the side compartment during the 40-h period of labeling or from diffusion of radioactive amino acid through the film of medium between the compartments.

Migration of non-neuronal cells through the film of medium separating the compartments proved to be a slow process that appeared to stop after a few days in culture under the experimental conditions employed. The first background cells in the side compartments were usually observed at 3 d, and never before 48 h, after plating. Taking into account a short lag phase after plating, we estimate the time for the passage of a cell through the film of medium to be somewhere around 48 h. After a few days in culture, the appearance of new non-neuronal cells in the side compartments virtually ceased, possibly due to blockade of the medium passage by sessile cells. Indeed, when non-neuronal cells from spinal cord or from DRG were grown in the center compartment in the absence of neurons, and when labeling was done according to the standard procedure, the radioactivity incorporated into the proteins of the side compartment cells was scattered

¹ Abbreviations used in this paper: DRG, dorsal root ganglia; VSC, ventral spinal cord; MEM, Eagle's minimal essential medium.

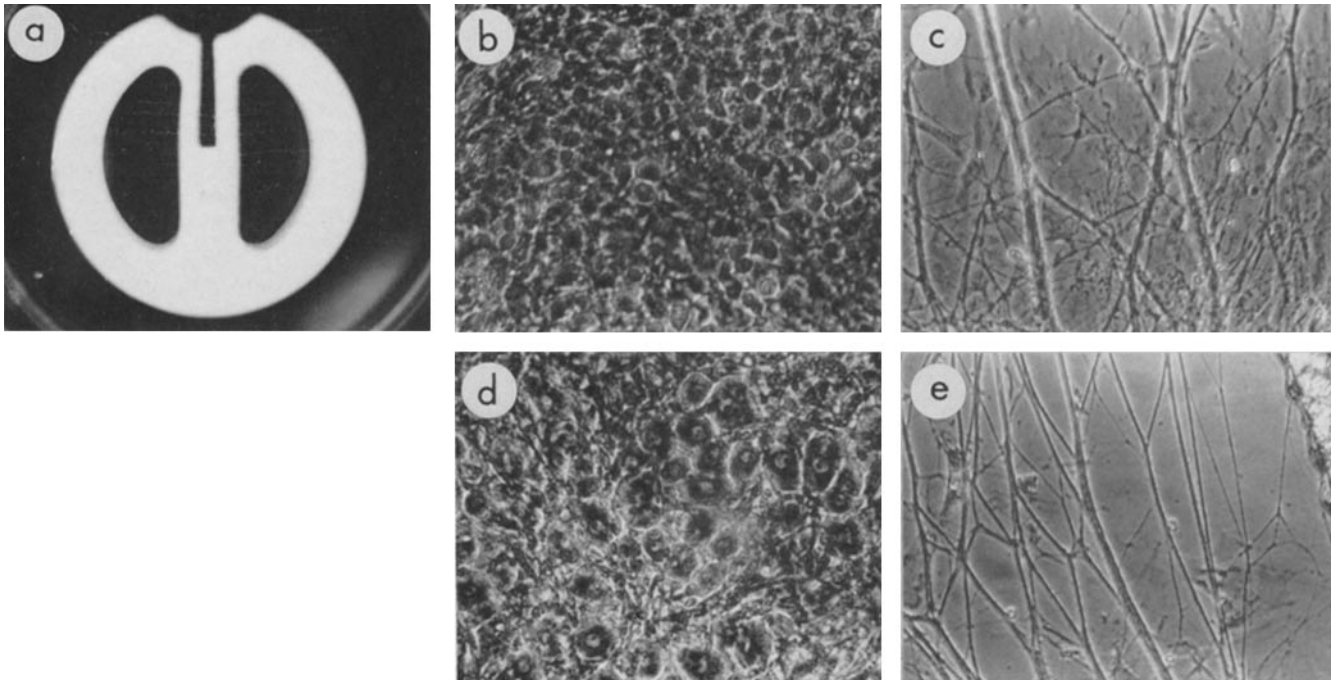


FIGURE 1 Three-compartment cell-culture system and morphological aspects of cultured VSC and DRG cells and their axons. (a) The Teflon three-compartment system devised by Campenot (16, 17) as seen from above, placed in a 35-mm Falcon cell culture dish. (b and d) Phase contrast photomicrographs of VSC and DRG, respectively, grown in the center compartment for ~10 d. (c) Phase contrast photomicrograph of the axonal fascicles of VSC neurons as found in the side compartments after 1 wk in culture. (e) Axons of DRG neurons in a side compartment after 1 wk in culture. (a) $\times 2.6$; (b and d) $\times 360$; (c and e) $\times 220$.

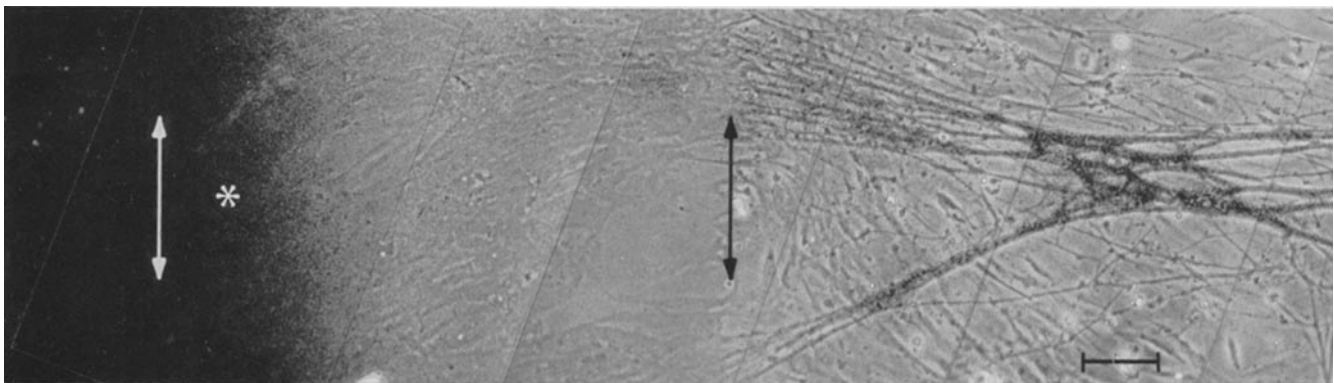


FIGURE 2 Radioautography of cells grown in the compartmental cell-culture system. The arrows indicate the area of the film of medium separating the center compartment from the side compartment. Silver grains are seen over cells of the center labeled compartment, over a narrow zone (asterisk) in the separating area adjacent to the center compartment (see Results and Discussion), and over axon fascicles in the side compartment. Phase contrast optics. Bar, 0.1 mm. $\times 90$.

around the same values as when the side compartment cells were exposed to side compartment medium containing diffused radioactive label from a previous labeling procedure. Radioautography of the area separating the center and side compartments, after removal of the Teflon inset (Fig. 2), showed a diffuse grain density occurring only in a zone immediately adjacent to the central heavily labeled compartment. Approximately two-thirds of the separating area was essentially free of silver grains. In the side compartment, radioautographic label was confined to axon fascicles. These data strongly suggest that migration of heavily labeled non-neuronal cells from the center compartment during the period of labeling does not significantly contribute to the radioactivity found in proteins of the side compartment.

Radioautograms were also prepared from cultures that, after labeling, were subjected to the standard collection procedure. After sodium dodecylsulfate/ β -mercaptoethanol dissolution of the cellular material of the side compartments, a broad band of non-neuronal material of the area separating the center and the side compartments remained intact. The cells in the central part of this separating area were neither labeled by radioactive amino acid from the center compartment nor accessible to the sodium dodecylsulfate/ β -mercaptoethanol solution from the side compartment (data not shown).

Some diffusion of radioactive amino acid through the film of medium was expected to occur as a feature inherent in the design of this system and was considered as a possible source of contamination of the axonally transported neuronal pro-

teins. Indeed, a minimal concentration of radioactive label ($\sim 20 \mu\text{Ci/ml}$) was detected in the medium of the side compartments following an incubation period of 40 h, when a concentration of 1 mCi/ml [^{35}S]methionine was used in the center compartment. The incorporation of radioactive methionine into proteins synthesized by non-neuronal cells of the side compartment was competitively reduced by the addition of excess unlabeled methionine (4 mM) to the medium of the side compartments for the period of labeling. The degree of contamination was determined by collecting medium from the side compartments of five plates after a 40-h labeling period and adding it for 40 h to the side compartments of the same number of fresh plates containing approximately the same number of axons and accompanying non-neuronal cells. The incorporation of radioactivity into trichloroacetic acid-precipitable material was found to be $<2\%$ of that obtained by the usual labeling procedure and did not evoke any spots of labeled proteins other than those for tubulin and actin, the most abundant cellular proteins, when subjected to 2-dimensional SDS PAGE (Fig. 3c). This system therefore ensured that all the proteins examined are derived from neurons since

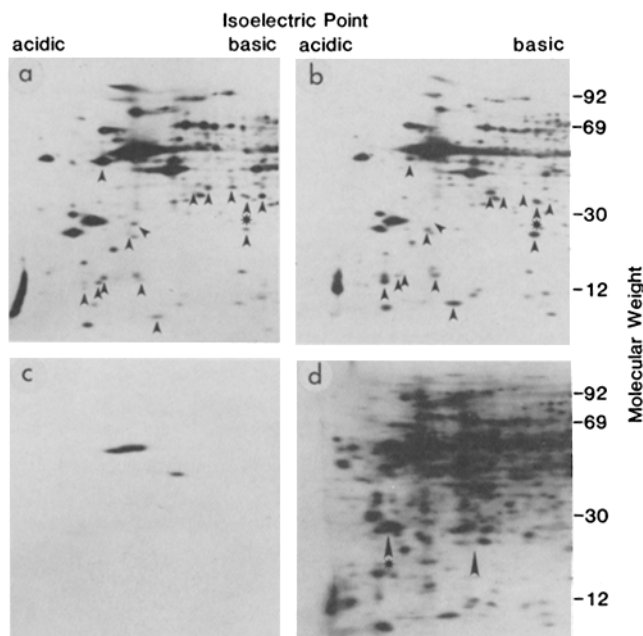


FIGURE 3 Two-dimensional electropherograms of the axonal proteins of VSC and DRG neurons (all panels 60% reduced). (a and b) Protein patterns of VSC neurons and DRG neurons, respectively. Each protein present in markedly different quantity in VSC axons and DRG axons is indicated by an arrowhead. The extrapolated location, relative to the surrounding spots, of absent or barely visible proteins, is indicated. The protein marked by the arrowhead with the asterisk is one of the proteins previously found to be synthesized with decreased abundance after co-culture of DRG axons with VSC cells and synapse formation (18). Values at right are molecular weights $\times 10^3$. (c) Control for contamination of the axonal proteins by labeled proteins derived from non-neuronal cells of the side compartments. After a labeling period of 40 h, the side compartment medium from five plates was collected and transferred to the side compartment of five fresh plates containing an equivalent number of axons and non-neuronal cells. (d) Protein pattern of cellular material of the center compartment of a DRG culture. A cluster of proteins that is absent from the axonal protein pattern is indicated by an arrowhead. The "landmark" spot used for approximate visual matching of the density of the three protein patterns is indicated by an arrowhead with an asterisk.

these are the only cells whose cell bodies are in the central, labeling compartment and whose axonal extensions are in the side, sampling compartment. Contamination by labeled proteins from the inevitable, and also variable, non-neuronal components of the cultured tissue is virtually excluded. However, no claim can be made as to the completeness of the representation of the axonal proteins: first, higher resolution techniques may permit detection of many more proteins on a single gel; second, the use of one amino acid for labeling certainly excludes certain proteins from being visualized; and third, possible synthesis of proteins within axons (24–29) may be dependent on the local availability of necessary amino acids.

The cell culture conditions were designed to compare two populations of neurons at a time of active axon outgrowth *in vitro*. Additionally, the cells to be cultured were dissected at an embryonic age at which active axon outgrowth was also known to occur *in vivo*. Most of the neuronal cells destined for the ventral horn withdraw from the mitotic cycle in the developing chick embryo during the third d (stages 17–18 of Hamburger and Hamilton; 30, 31). The birthdate of the bulk of the neuronal cells of the DRG, however, has been determined to occur between day 4.5 and 7.5 of embryonic age (32). Hence the VSC and the DRG were dissected on about the third and fourth days, respectively, after the majority of their neurons had entered the postmitotic phase and at a time of active axon outgrowth and synapse formation (33). The two neuronal populations used in the present study were, therefore, developmentally approximately equivalent at the time when they were placed in culture.

The two-dimensional fluorographic representations of the axonal protein patterns of VSC and DRG neurons are quite similar (Fig. 3, a and b). However, distinct, and in some cases qualitative, differences have been revealed. In three independent experiments we found seven proteins in the molecular weight range between 10,000 and 100,000 (where the major part of the cellular proteins is usually found), that were more abundantly expressed in VSC axons than in DRG axons. Also, seven other proteins in the same molecular weight range were expressed primarily in DRG axons. Possible mechanisms underlying the generation of these differences might include posttranslational modifications or changes in the rate of protein synthesis, degradation, or axonal transport.

The gel pattern of cellular material from the center compartment was, as anticipated, substantially more complex than that of the axons (Fig. 3d). Matching an arbitrary landmark spot for equal density in both gels (arrowhead with asterisk in Fig. 3d) revealed many more proteins in the cellular protein pattern than in the axonal protein profile. Sets of proteins not visible in the axonal protein patterns were evident (arrowhead, Fig. 3d), and some areas were crowded with spots close to confluence. It is impossible, however, to assign any of these proteins to a particular cell type or to a cellular compartment due to the complex composition of this cellular material. These fluorograms clearly illustrate the reduction in signal complexity obtained by the use of this compartmental cell-culture system for the study of axonal proteins.

Axons from different neuronal subsets have structural and functional features in common. The high degree of similarity observed between the axonal protein patterns of VSC and DRG certainly reflects these commonalities. However, there are a number of morphological and functional characteristics

that clearly distinguish axons of different neuronal populations from each other. In particular, a substantial proportion of cultured VSC neurons form cholinergic synapses with cultured myotubes (34), whereas the DRG neurons make noncholinergic synaptic connections with central neurons (35). During development, VSC and DRG neurons show distinct behavior with respect to axon outgrowth and establishment of synapses. Even in the light of the fact that neither cultured VSC (36) nor DRG (37) neurons are homogeneous populations, the different axonal proteins observed from these two enriched neuron populations are likely candidates for a role in the implementation of those specific functions that distinguish VSC and DRG axons from each other. One of the proteins, expressed with markedly higher abundance in DRG axons than in VSC (arrowhead with asterisk in Fig 3, *a* and *b*), has been previously identified as a protein that is diminished in DRG axons when they are co-cultured with cells from VSC under conditions in which synapse formation occurs (18). There is evidence for the involvement of particular features of the axonal surface in axonal fasciculation (38, 39), pathfinding (40), and specific synapse formation (41). Studies are now in progress to identify those cell-type specific proteins that are exposed on the cell surface and to determine whether they subserve such topogenetic functions.

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