Laminin Promotes Neuritic Regeneration from Cultured Peripheral and Central Neurons

MARSTON MANTHORPE, EVA ENGVALL,* ERKKI RUOSLAHTI,* FRANK M. LONGO, GEORGE E. DAVIS, and SILVIO VARON

*Department of Biology, School of Medicine, University of California at San Diego, La Jolla, California 92093; and *Cancer Reseach Center, La Jolla Cancer Research Foundation, La Jolla, California 92037*

ABSTRACT The ability of axons to grow through tissue in vivo during development or regeneration may be regulated by the availability of specific neurite-promoting macromolecules located within the extracellular matrix. We have used tissue culture methods to examine the relative ability of various extracellular matrix components to elicit neurite outgrowth from dissociated chick embryo parasympathetic (ciliary ganglion) neurons in serum-free monolayer culture. Purified laminin from both mouse and rat sources, as well as a partially purified polyornithine-binding neurite promoting factor (PNPF-1) from rat Schwannoma cells all stimulate neurite production from these neurons. Laminin and PNPF-1 are also potent stimulators of neurite growth from cultured neurons obtained from other peripheral as well as central neural tissues, specifically avian sympathetic and sensory ganglia and spinal cord, optic tectum, neural retina, and telencephalon, as well as from sensory ganglia of the neonatal mouse and hippocampal, septal, and striatal tissues of the fetal rat. A quantitative in vitro bioassay method using ciliary neurons was used to (*a*) measure and compare the specific neurite-promoting activities of these agents, (*b*) confirm that during the purification of laminin, the neurite-promoting activity co-purifies with the laminin protein, and (c) compare the influences of anti-laminin antibodies on the neurite-promoting activity of laminin and PNPF-1. We conclude that laminin and PNPF-1 are distinct macromolecules capable of expressing their neurite-promoting activities even when presented in nanogram amounts. This neurite-promoting bioassay currently represents the most sensitive test for the biological activity of laminin.

Neurons in the peripheral nervous system (PNS)[1] can successfully regrow transected axons in vivo, often achieving substantial recovery of function (1), whereas central nervous system (CNS) neurons usually regenerate less well, if at all (2). Two requisites for such regeneration are that the injured neurons remain alive and that they are presented with a favorable microenvironment through which their regrowing neurites can advance. Implanted segments of peripheral nerve have been shown to provide a suitable microenvironment for axonal regeneration from both PNS and CNS neurons (3, 4). This axonal regrowth takes place upon cell surfaces or within the extracellular matrix (ECM) with which the advancing growth cone makes contact. ECM constitutes and particularly ECM glycoproteins may, therefore, constitute a family of contact-operating neurite-promoting agents.

A complementary approach to the study of axonal regeneration has been through the use of neuronal models in vitro. Neurons in culture also have two requirements for neuritic regrowth, namely, trophic factors that support the survival of neurons (5, 6), and substances that act specifically as neurite-promoting factors (7–13). It is reasonable to suppose that the latter agents may relate to the ECM constituents. The use of more highly adhesive substrata, such as tissue culture plastic (TCP) coated with polycationic substances (polylysine; polyornithine or PORN) provide for a rapid cell attachment even in the absence of added ECM constituents. Previous

[1] *Abbreviations used in this paper*: CNS, central nervous system; DME, Dulbecco's Modified Minimal Essential Medium containing $NaHCO_3$ (to 26.4 mM), glutamine (to 2 mM), and penicillin (100 U/ml); ECM, extracellular matrix; PNPF, polyornithine-binding neurite-promoting factors; PNS, peripheral nervous system; PORN, polyornithine; PORN-TCP, PORN-coated TCP; TCP, tissue culture plastic.

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 DECEMBER 1983 1882-1890

studies have demonstrated the presence, within culture media exposed to glial and other cells, of high molecular weight agents designated as polyornithine-binding neurite promoting factors or PNPF (9, 13–16). These agents have been shown to attach and confer to PORN-coated plastic surfaces neurite-promoting activity for dissociated PNS and CNS neurons (16–18). Fibronectin, a prominent component of the ECM, cell surfaces, and plasma, has been reported to stimulate neurite outgrowth from retinal cell reaggregate cultures (18). Very recently laminin, a glycoprotein component of the basal lamina (19), was reported to confer to tissue culture plastic surfaces a neurite-promoting activity for explanted human fetal sensory ganglia (20).

In this study we have examined the ability of several purified ECM components to confer neurite-promoting activity to plastic or PORN-coated plastic surfaces and we show that laminin from both rat and mouse sources is an extremely potent PNPF for both peripheral and central neurons. Fibronectin has a relatively weaker neurite-promoting activity using a peripheral neuron bioassay, while two proteoglycans tested lack detectable activity. Furthermore, we present evidence that rat laminin is distinct from partially purified PNPF derived from rat RN22 Schwannoma cells.

MATERIALS AND METHODS

Reagents: Culture medium (DME-N1) was Dulbecco's Modified Minimal Essential Medium containing $NaHCO_3$ (to 26.4 mM), glutamine (to 2 mM), and penicillin (100 U/ml) (DME; Gibco Laboratories, Grand Island, NY) and containing the N1 supplement (21). Double-strength medium contained the N1 supplement at double its normal concentration. PORN (molecular weight $\approx 3 \times 10^4$; Sigma P6012, Sigma Chemical Co., St. Louis, MO), ovalbumin, and hyaluronic acid were obtained from Sigma Chemical Co. PNPF-1 was partially purified from serum-free RN22 Schwannoma conditioned medium (33). Laminin was purified from rat yolk sac tumor or conditioned medium from mouse endodermal cell line PF HR-9 (22). Other ECM-related materials were fibronectin from rat plasma (23), a chondroitin sulfate proteoglycan from rat yolk sac tumor (24), and a heparan sulfate proteoglycan from a rat hepatoma (25). Heparin was kindly provided by Dr. Ulf Lindahl, Uppsala, Sweden. Protein was determined using the method of Bradford (26).

Neuronal Cell Dissociation: Dissociates from embryonic day 8 (E8) chick ciliary ganglia were prepared as described (27), except that the posttrypsin washes and trituration were carried out with 1% ovalbumin in DME and the cells were diluted in DME. The same modifications were applied to previously described procedures for the preparation of cell suspensions from E11 chick paravertebral sympathetic ganglia (28), E8 chick and neonatal mouse dorsal root ganglia (28), E4 chick lumbar spinal cord (16), E8 chick telencephalon (21), optic lobe (29), and neural retina (17), and 18-d fetal rat hippocampus, septum, and striatum (30). All dissociated cell suspensions were diluted to 40,000 cells/ml, except spinal cord which was diluted to 20,000/ml.

Substratum Preparation: All experiments used 6-mm microwells in 96-well culture plates (Costar 3596; Bellco Glass, Inc., Vineland, NJ). Each well was first incubated overnight at 25°C with 50 μl of PORN solution (0.1 mg/ml in 15 mM borate buffer, pH 8.4; Sigma Chemical Co.), followed by 3 × 50-μl water washes. The PORN-treated wells then were supplied with 50 μl of the materials to be tested for neurite-promoting activity (serially diluted in PBS, pH 7.0), incubated for 2 h at 37°C, and washed once with 100 μl of PBS containing 1% ovalbumin.

Culture Preparation and Analysis: The pretreated culture wells received 50 μl of double-strength culture medium followed by 50 μl of cell suspension in DME containing the required neuronotrophic factor: 100 Trophic Units per milliliter of eye-derived ciliary neuronotrophic factor for ciliary ganglia (31), 20 Biological Units per milliliter of 7S nerve growth factor (32) for E11 chick sympathetic ganglia, E8 chick and neonatal mouse dorsal root ganglia (28), or 25% (vol/vol in DME) rat astroglial conditioned medium, which had been depleted of endogenous PNPF activity by passage through a PM10 membrane (Amicon Corporation, Danvers, MA), for all of the CNS cultures (33, 34). It should be emphasized here that under all of the culture conditions reported in this study no neurons would survive without the addition of an adequate and appropriate supply of neuronotrophic factors. After 24-h incubation (37°C, 5% CO_2-air), the cultures were fixed and neuronal numbers and percentages of neurons bearing neurites greater in length than one somal diameter were determined using phase contrast microscopy as described (13, 35, 36). For photomicrographs, the neurons were stained using silver nitrate and photographed using phase contrast optics, a procedure that enhances the visibility of neurites.

Immunochemical Analysis: Rabbit antiserum to purified rat laminin was prepared and characterized as described (22). The antiserum was tested for its ability to block rat laminin, mouse laminin, or rat PNPF-1 as follows. A constant amount (1 μg/ml, 50 μl) of laminin or PNPF-1 was presented for 2 h at 37°C to the PORN-treated wells followed by washes as indicated above. Then, 50 μl of serially diluted immune or preimmune serum were presented to wells (37) for 2 h at 37°C, and the wells were washed, seeded with ciliary ganglion neurons, and evaluated for their ability to elicit neurite production.

RESULTS

Selected Extracellular Matrix-Related Materials Support Neuronal Maintenance and Stimulate Neurite Regeneration from Cultured Ciliary Ganglion Neurons

NEURONAL MAINTENANCE: Table I compares the effects, on ciliary ganglion neurons cultured in serum-free conditions, of rat RN22 Schwannoma–derived PNPF-1 and various ECM-related agents presupplied to either TCP or PORN-coated TCP (PORN-TCP) substrata. Despite the presence of an adequate supply of the ciliary neuronotrophic factor in the medium, very few neurons could be maintained on TCP that had been unexposed (Table I, *A*), or exposed to most of the test agents (Table I, *B* and *C*). However, both fibronectin and laminin, particularly when presented to TCP at the highest concentration shown, 10 μg/ml, were able to sustain several-fold more neurons than unexposed TCP (Table I, *D*). In contrast, both untreated and treated PORN-TCP supported a relatively constant and higher neuronal number representing 60–85% of the number of neurons seeded (i.e., 1,000 neurons/well). The one exception to this was the heparin-exposed PORN-TCP wells (Table I, *C*) in which less than half of this number of neurons was sustained. No effort was made in this study to seek the explanations (neuron-substratum adhesiveness, neuronal survival regulation, toxicity, etc.) for such effects of different substrata on neuronal maintenance.

NEURITE REGENERATION: Partially purified PNPF-1 (13) was able to elicit a distinct but modest neurite growth on TCP, and this only at relatively high concentrations (10 μg/ml, Table I, *B*). However, on PORN-TCP, PNPF-1 recruited nearly all of the neurons present into neurite production even when presented in very low amounts. Neither of the proteoglycans nor hyaluronic acid (Table I, *C*) elicited neurite growth from ciliary ganglion neurons and in fact they appeared to inhibit the modest growth normally appearing on untreated PORN-TCP. Purified rat fibronectin conferred some neurite-promoting activity to TCP and to a lesser extent to PORN-TCP, particularly when presented at relatively high levels (10 μg/ml; Table I, *D*).

Rat and mouse laminin conferred to both TCP and PORN-TCP considerable neurite-promoting competence (Table I, *D*). This activity appeared to parallel their neuron-sustaining ability on the TCP substratum and both activities were essentially lost when the laminins were presented to TCP at 0.1 μg/ml. In contrast, even this low laminin concentration was able to confer to PORN-TCP substrata near maximal neurite-promoting activity.

Thus the PORN-TCP surface offered at least four advantages over TCP, namely (*a*) the PORN substratum sustained

TABLE I

Influence of PNPF-1 and Purified Extracellular Matrix Materials on Neurite Outgrowth from Chick Embryo Ciliary Ganglion Neurons Cultured on Plastic Substrata

Substratum exposed to:	Test material added as 50 μl/well	TCP substratum: Untreated		Coated with PORN	
		No. of neurons*	With neurites	No. of neurons*	With neurites
	μg/ml		%		%
A. Buffered saline (control)	0	100	0	770	10
B. Rat PNPF-1	10	50	15	730	94
	1	90	2	750	94
	0.1	90	0	640	12
C. Heparan sulfate proteoglycan	10	100	0	840	0
Chondroitin sulfate proteoglycan	10	100	0	710	0
Heparin	10	110	0	380	0
Hyaluronic acid	10	110	0	730	7
D. Rat fibronectin	10	430	63	600	28
	1	320	16	600	14
Rat laminin	10	470	86	770	99
	1	150	28	760	96
	0.1	110	0	680	84
Mouse laminin	10	360	86	650	98
	1	230	74	790	94
	0.1	90	0	660	86

* This represents the total number of surviving neurons per culture well. The number of cells originally seeded was 2,000, about half of which were neurons.

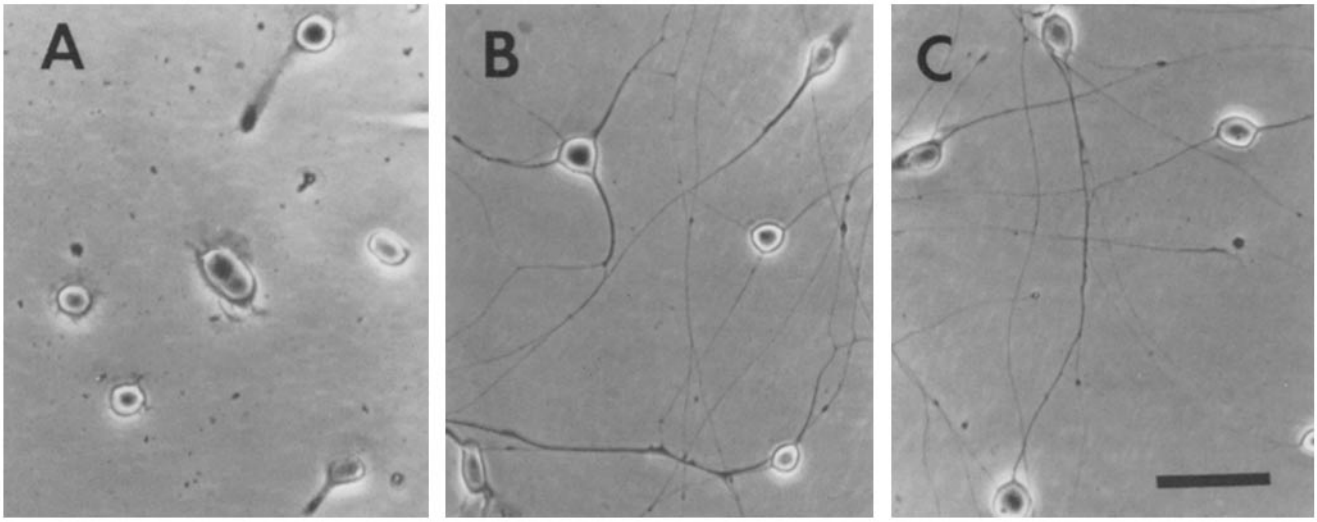

FIGURE 1 The promotion of neurite growth from chick embryo ciliary ganglion neurons by laminin and partially purified PNPF-1 from rat Schwannoma. Dissociated E8 chick ciliary ganglionic neurons were cultured for 24 h in serum-free medium supplemented with ciliary neuronotrophic factor on three different substrata as described in Materials and Methods. The cultures were then washed, fixed, stained using silver impregnation, and photographed. The substrata were (*A*) PORN-TCP; (*B*) rat laminin- (1 μg/ml) coated PORN-TCP; (*C*) rat PNPF-1- (1 μg/ml) coated PORN-TCP. Bar, 50 μm. × 260.

a relatively high and constant neuronal number, independent of further substratum pretreatments, (*b*) the neurite-promoting activity of materials used for subsequent treatments could be measured with greater sensitivity, and (*c*) neurite-promoting activity could be measured independent from cell-sustaining activity. In addition, (*d*) the distribution of the neurons over the culture surface was more uniform on PORN-TCP, making replicate diametral neuronal count values more consistent. For these reasons PORN-TCP was used to examine in more detail the laminin and PNPF-1 responses.

MORPHOLOGY OF CILIARY GANGLION NEURONS CULTURED IN SERUM-FREE MEDIUM ON PORN-TCP COATED WITH LAMININ OR PNPF-1: Some ciliary ganglion neurons exhibited a definite, although modest neuritic growth on PORN-TCP (Fig. 1 *A*) when cultured in serum-free medium, in contrast to our earlier studies (13, 14) in which serum was routinely used. These neurites seldom exceeded five somal diameters and were present on 10% or less of the neuronal population (cf. Table I). However, when the PORN-TCP was exposed to either PNPF-1 or laminin, virtually all of the neurons exhibited neurite growth (Fig. 1, *B* and *C*). The morphology of the neurites appeared indistinguishable in the two cases; neurite length was usually greater than 20 somal diameters and sometimes extended over 1.0 mm.

QUANTITATION OF THE NEURITE-PROMOTING ACTIVITY OF LAMININ: We have previously developed a quantitative bioassay for the RN22 Schwannoma–derived

PNPF and have used this short-term assay to monitor its purification (13). Here we have used a similar assay to (*a*) estimate the specific neurite-promoting activity (units of activity per milligram of protein) of rat and mouse laminin, (*b*) compare the specific activities of the laminins with that of the partially purified rat PNPF-1, and (*c*) confirm that during the purification of laminin the neurite-promoting activity copurifies with the laminin protein. We have also used the assay to show that anti-laminin antibodies inhibit quantitatively the neurite-promoting activities of laminin.

Fig. 2 shows the relationship between neurite promotion and dosages of purified rat laminin, mouse laminin, or partially purified rat PNPF-1 using ciliary ganglionic neurons in serum-free culture medium on PORN-TCP. Both laminins and PNPF-1 can recruit up to nearly 100% of the ciliary ganglion neurons into neuritic production when used for PORN-TCP pretreatment at concentrations of 1 μg/ml. This recruitment decreases as the pretreatment concentration is decreased. The limits of reliable detection (i.e., ~20% neuritic growth) are reached when the laminins and PNPF-1 are presented to the wells at 25 and 200 ng/ml, respectively. The half-maximal responses (i.e., those elicited by 1 neurite-promoting unit or NPU per ml) are reached when 50 ng/ml of laminin or 300 ng/ml of PNPF-1 is presented to the PORN-TCP well. Thus it is possible to derive a *specific activity* (NPU/mg protein) for the rat laminin, mouse laminin, and rat PNPF-1 of, respectively, about 20,000, 20,000, and 3,300 NPU/mg of protein.

Co-purification of Rat Laminin Antigen and Rat Laminin-induced Neurite-Promoting Activity

The quantitative bioassay shown in Fig. 2 was used to monitor neurite-promoting activity in sequential fractions

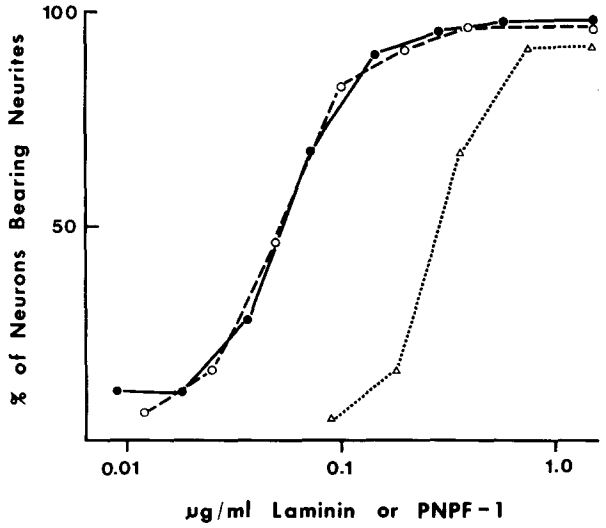

FIGURE 2 In vitro titration of the neurite-promoting activity of laminin and PNPF-1 for ciliary ganglion neurons. Serial dilutions of purified rat laminin (solid line) and mouse laminin (dashed line) and partially purified rat Schwannoma-derived PNPF-1 (dotted line) were presented for 2 h at 37°C as 50 μl to 6-mm diam PORN-TCP microwells, the wells were washed, and ciliary ganglion neurons were added for 24 h as described in Materials and Methods. The cultures were then fixed and the proportion of the neuronal population having at least one neurite greater in length than one somal diameter was determined. The total neuronal number per well ranged from 580 to 700 under all the conditions tested.

TABLE II

Co-purification of Rat Laminin Antigen and Rat Laminin-induced Neurite-promoting Activity for Ciliary Ganglion Neurons

Fraction*	Specific activities: mg laminin/mg protein‡ ELISA	Specific activities: Neurite promoting§ U/mg protein
3.5 M NaCl	0.02	1,700
0.5 M NaCl	0.41	16,500
Sepharose 6B pool	0.71	34,000
Heparin-Sepharose affinity chromatography pool	0.97	38,000

* Laminin was purified from rat yolk sac tumor tissue as described (22).
‡ As determined by Sandwich enzyme-linked immunoadsorbent assay (22) and by a Coomassie Blue binding assay (26).
§ As determined using the bioassay described in Fig. 2 and the text.

obtained during the course of laminin purification from rat yolk sac tumor (22). Table II compares specific activities measured in such fractions using an enzyme-linked immunoadsorbent assay and the neurite promotion bioassay. The specific activity for neurite promotion increased progressively with the progression of laminin purification, thereby providing evidence that laminin itself, rather than a nonlaminin contaminant, is responsible for the neuritic effect. The ratio of the two data columns (i.e. U/mg laminin) decreased from ~85,000 in the 3.5 M NaCl fraction to 34,000 in the final product. This decrease could be due to a loss of the neurite-promoting activity of laminin incurred during the purification procedure. Three different rat laminin preparations purified to homogeneity by electrophoretic criteria have yielded specific activities of 18,000, 20,000, and 38,300 NPU/mg.

EFFECTS OF ANTI-LAMININ ANTIBODIES ON THE NEURITE-PROMOTING ACTIVITY OF LAMININ AND PNPF-1: We next studied the ability of rabbit anti-rat laminin antibodies to block the neurite-promoting activity of rat and mouse laminin and rat PNPF-1. Each neurite-promoting agent was first presented to the PORN-TCP wells at 1 μg/ml concentrations (i.e., enough to assure a maximal neurite response—see Fig. 2) followed by a second 2-h incubation with serial dilutions of anti-rat laminin antiserum (50 μl/well). The wells were then washed and examined for their retained ability to promote neuritic growth. The results are shown in Fig. 3.

Despite the similarity of their biological activities the laminin and PNPF-1 are not identical molecules. Anti-rat laminin serum blocks the neurite-promoting activity of purified rat and mouse laminin but not that of rat PNPF-1. This particular antiserum was also capable of blocking the neurite-promoting activity of crude conditioned medium from the mouse cell line PF-HR9, which contains laminin (38), but incapable of blocking a similar activity in crude Schwannoma-conditioned medium as well as in conditioned media from purified cell cultures of rat astroglia (39), oligodendroglia (40), C-6 glioma (33), and mouse Schwann cells (41), all of which contain PNPF (15) (data not shown). Rabbit antiserum to rat fibronectin and normal rabbit serum did not affect the activity of either the laminins or PNPF-1 (data not shown).

Laminin and PNPF-1 Stimulate Neuritic Regeneration from Both Peripheral and Central Neurons in Culture

It has been previously demonstrated that a variety of PNS

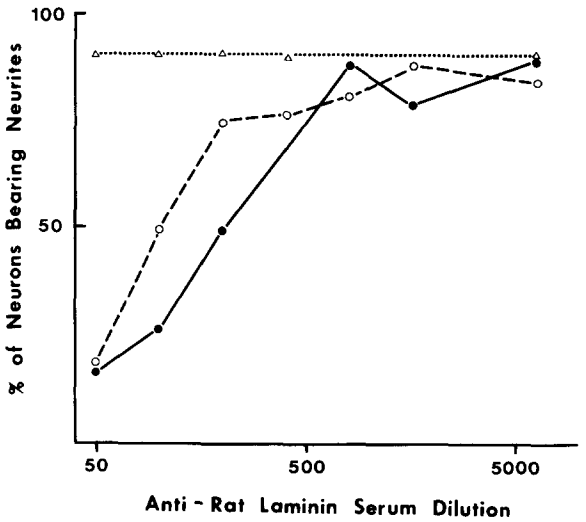

FIGURE 3 Immunochemical distinction between laminin and PNPF-1. Rabbit anti-rat laminin serum was tested for its ability to block the neurite-promoting activity of purified rat laminin (solid line), mouse laminin (dashed line), or partially purified rat PNPF-1 (dotted line). The laminins and PNPF-1 were presented to PORN-TCP at 1 μg/ml (enough to elicit a maximal neurite response, see Fig. 2) followed by the exposure of the substratum to the indicated serial dilution of the antiserum. The wells were then washed and analyzed for their ability to promote neurite growth from ciliary ganglion neurons as in Fig. 2. Note that the activity of rat laminin was more readily inhibited than that of mouse laminin activity and that PNPF-1 activity was not blocked by laminin antiserum. The reciprocal of the serum dilution is shown.

neurons (13, 14, 15) as well as some spinal cord (16) and retinal neurons (17) will increase neurite production on PORN-TCP in response to pretreatment of PORN-TCP with PNPF-containing RN22 Schwannoma–conditioned media. We have extended these observations, using partially purified PNPF-1, to a variety of avian and rodent peripheral and intrinsic central neurons and we have compared the response of all of these test neurons to that produced using purified rat laminin.

Fig. 4 illustrates the morphologies of E11 chick sympathetic and neonatal mouse dorsal root ganglionic neurons cultured on untreated PORN-TCP (Figs. 4, *A* and *D*), laminin-treated PORN-TCP (Fig. 4, *B* and *E*) and PNPF-1-treated PORN-TCP (Fig. 4, *C* and *F*). Both neuronal types exhibited dramatic neurite production in response to both laminin and PNPF-1 in a manner very similar to that already presented for ciliary ganglion neurons (cf. Fig. 1). Mouse sensory neurons appeared to produce some neurites on untreated PORN-TCP but these neurites were generally short, thick, and often grew near the perimeter of the neuronal soma (Fig. 4 *D*).

Fig. 5 illustrates the morphologies of E8 chick cerebral cortex (telencephalon) and 18-d fetal rat hippocampal neurons cultured on the same three substrata as shown in Fig. 4. The neurons from both of these tissue sources, previously identified as such by the use of tetanus toxin binding (30, 34), produced little, if any, neuritic growth on PORN-TCP, and the somata often appeared circular and flattened (Fig. 5, *A* and *D*). In contrast, treatment of the PORN-TCP substratum with either laminin (Fig. 5, *B* and *E*) or PNPF-1 (Fig. 5, *C* and *F*) elicited extensive neuritic growth from both chick and rat neurons.

Quantitative data for neuronal maintenance and neuritic responses to both laminin and PNPF-1 using selected neuronal cell types from avian and rodent peripheral and central nervous tissues are shown in Table III. The number of neurons maintained over the 24-h culture period was essentially the same for a given neuronal cell type when tested on either substratum. However, in the absence of an adequate supply of the appropriate neuronotrophic factor (see Methods), none of these neurons survive for even 24 h regardless of the substratum provided (14, 16, 17, 27, 30). On PORN-TCP, pretreated with only saline as the control, the proportion of neurite-bearing neurons was always <10%, with the one exception of mouse sensory neurons. As noted above, however, (i.e., Fig. 4 *D*), this growth was greatly restricted in length and frequency. Both laminin and PNPF-1 presented to the substratum at 1 μg/ml, elicited considerable neuritic extension from all of the neuronal types tested. It remains to be determined whether each of these neuronal types exhibits a dose-response to laminin and PNPF-1 that is similar to that previously shown for ciliary ganglion neurons (cf. Fig. 2).

DISCUSSION

The data presented here demonstrate that laminin is an extremely potent neurite-promoting agent in vitro. Laminin can exert its action when anchored to either polyornithine or TCP substrata, although it is orders-of-magnitude less effective with plastic. Laminin stimulates neuritic outgrowth from a variety of avian and rodent neurons derived from both peripheral and central neural tissues. Neurite promotion can be achieved at half-maximal levels by substratum pretreatments with 50 ng/ml or less of either rat or mouse laminin. Assuming a molecular weight of 10^6 for laminin (42), this represents a concentration of 4×10^{-11} M. It should be pointed out that the 50 ng/ml of laminin that elicits a half-maximal neurite response is an operational value and does not necessarily reflect the amount of laminin to which the neurons actually respond. Each culture well receives (e.g. for half-maximal effects) 0.05 ml (or 2.5 ng) of laminin, and possibly only a very small fraction of this will eventually be available to the neurons. This is because (*a*) the culture well bottom on which the neurons attach represents only 25% of the total inside well area exposed to the laminin, (*b*) all of the laminin presented may not bind to the PORN-TCP surface, and (*c*) being seeded at a low cell density, the neurons or their neurites or growth cones will have an opportunity to contact only a fraction of the total area of the laminin-bound culture surface. Thus, the amount of laminin to which the neurons actually respond by neuritic production may be far lower than the amount originally presented to the culture well. Irrespective of these considerations, the bioassay for neurite-promoting activity constitutes a more sensitive test for the biological activity of laminin than previous assays that measure its effects on the adhesion or differentiation of other types of cells (43–47).

The attribution of neurite-promoting activity to laminin is supported by (*a*) the high potency of the laminin preparations, (*b*) the progression of specific neurite-promoting activity during the purification process which increases along with that of laminin immunoreactivity, and (*c*) the blocking effect of anti-laminin antibodies. That the activity is associated with laminin itself is also supported by the fact that laminins prepared from different sources using different methods are active. We used both mouse and rat laminin and, after this work was completed, a report by Baron-van Evercooren et al.

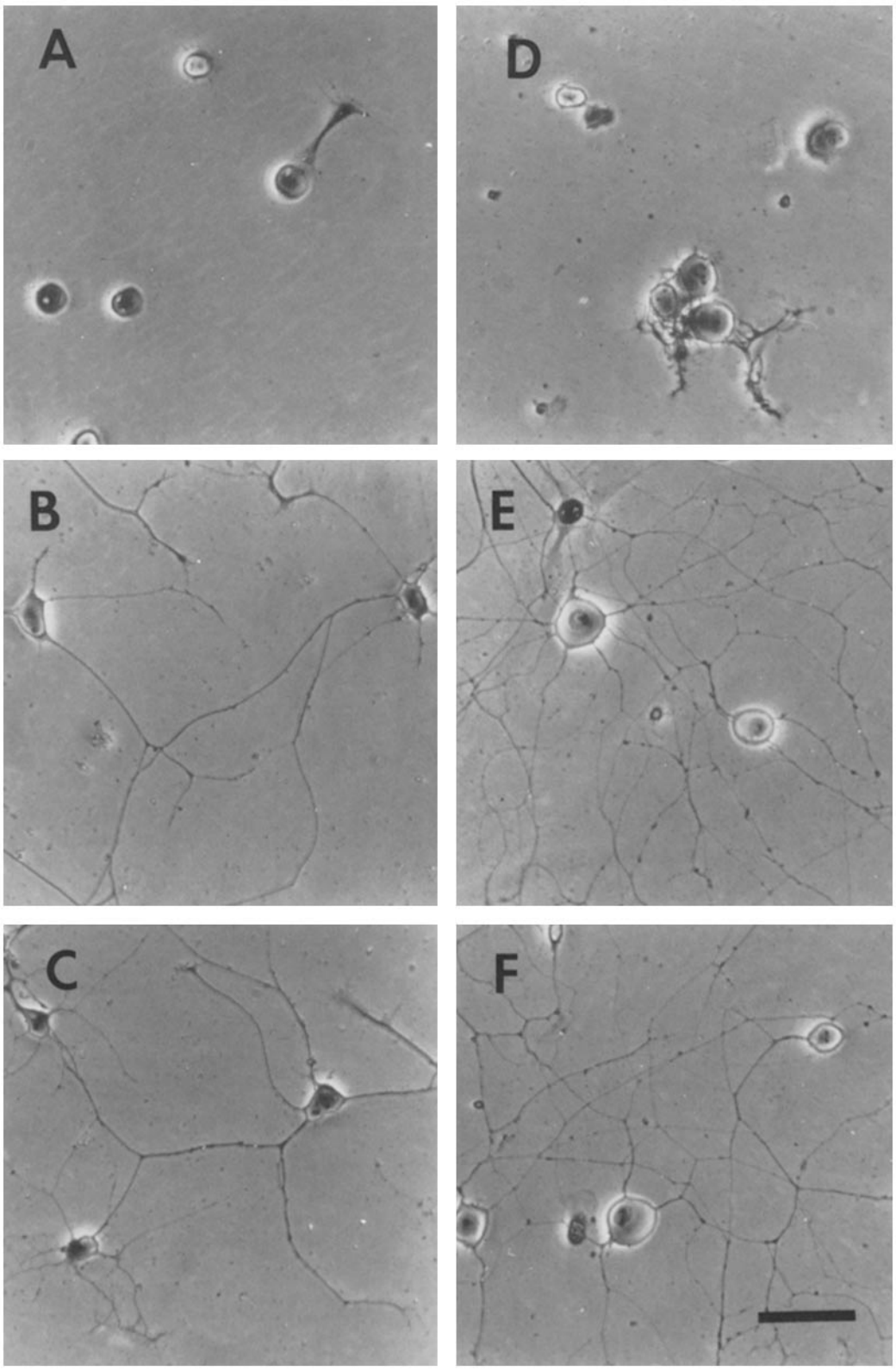

FIGURE 4 Stimulation of neurite regeneration from avian and rodent *PNS* neurons cultured on substrata treated with laminin and PNPF-1. Dissociated 11-d-old chick embryo sympathetic neurons (*A–C*) or neonatal mouse sensory neurons (*D–F*) were cultured for 24 h in serum-free medium supplemented with nerve growth factor on three different substrata as described in Materials and Methods. The cultures were then washed, fixed, and stained using silver impregnation. The substrata were *A* and *D*, PORN-TCP; *B* and *E*, rat laminin- (1 μg/ml) coated PORN-TCP; C and *F*, rat PNPF-1- (1 μg/ml) coated PORN-TCP. Bar, 50 μm. × 260.

(20) appeared attributing neurite-promoting activity to laminin prepared from a mouse tumor different from the one used here. Moreover, we have recently observed (48) that neurite-promoting activity is also conferred to PORN-TCP by fragments of human laminin, obtained from peptic digests of human placenta and purified on the basis of affinity for an immobilized monoclonal antibody to human laminin.

On plain TCP, both fibronectin and laminin had to be presented at relatively high concentrations (1–10 μg/ml) in order to elicit even a modest neurite-promoting effect (Table I). On PORN-TCP, however, fibronectin treatment elicited very little neurite growth, and this only at concentrations 1,000-fold higher than the laminin concentrations needed for the same effect. To promote neurite regeneration in the

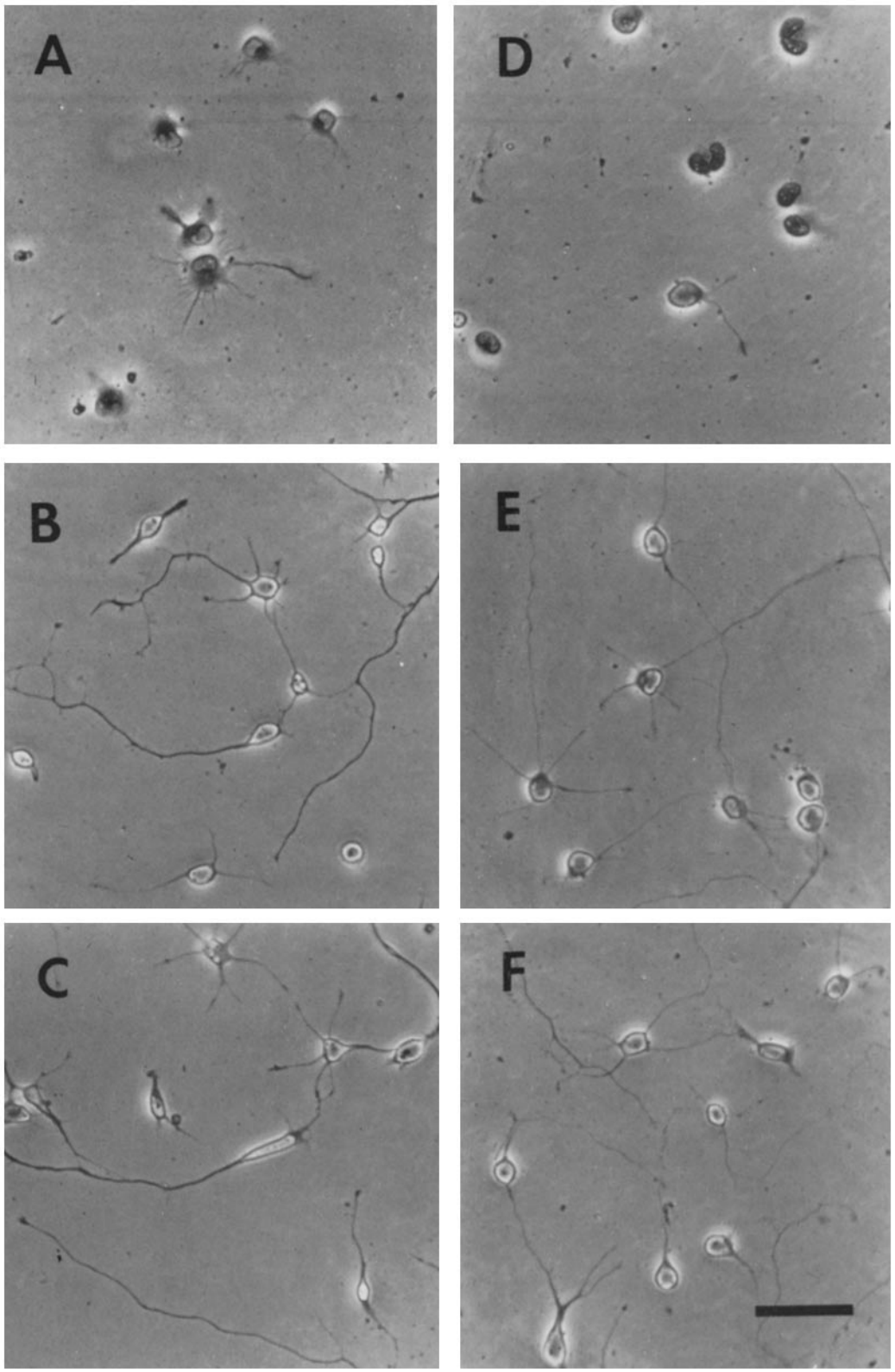

FIGURE 5 Stimulation of neurite regeneration from avian and rodent *CNS* neurons cultured on substrata treated with laminin and PNPF-1. Dissociated 8-d-old chick embryo cerebral cortex neurons (*A–C*) or 18-d-old fetal rat hippocampal neurons (*D–F*) were cultured on the three substrata for 24 h in serum-free medium supplemented with ultrafiltrate from rat astroglial cell conditioned medium as described in Materials and Methods. The cultures were then washed, fixed, and stained using silver impregnation. The substrata were *A* and *D*, PORN-TCP; *B* and *E*, rat laminin- (1 μg/ml) coated PORN-TCP; *C* and *F*, rat PNPF-1- (1 μg/ml) coated PORN-TCP. Bar, 50 μm. × 260.

present bioassay, an agent must be endowed with both neurite-promoting competence and adequate affinity for the PORN-TCP substratum. Thus, the relatively low activity of fibronectin may derive from insufficient binding to polyornithine rather than from actual lack of neurite-promoting capabilities. Conversely, laminin may be a weaker neurite promoter on TCP because it does not allow adequate adhesion of the neurons. The PORN-TCP allows efficient adhesion of the neurons, and laminin, which binds to polyornithine, can then promote neurite regeneration.

Our results regarding the relative inefficiency of fibronectin to promote neurite growth are in agreement with previous reports. Baron-van Evercooren et al. (20) observed neurite-promoting activity from human fetal sensory ganglionic ex-

TABLE III

Induction of Neurite Elongation from Cultured Peripheral and CNS Neurons by PORN-TCP–Bound Laminin and PNPF-1

Neuronal source	No. of neurons* (range)	Percent with neurites on PORN-TCP pretreated with: Saline (control)	Laminin (1 μg/ml)	PNPF-1 (1 μg/ml)
PNS ganglia				
E8 chick ciliary	700–840	6	97	97
E8 chick dorsal root	910–1,070	2	59	82
E11 chick sympathetic	830–970	1	70	70
Neonatal mouse dorsal root	580–600	45	93	83
CNS tissue				
E4 chick lumbar spinal cord	450–470	3	49	83
E8 chick telencephalon	1,440–1,550	7	52	69
E8 chick optic tectum	1,440–1,750	4	46	45
E8 chick neural retina	1,060–1,210	2	43	81
E18 rat hippocampus	1,110–1,390	8	80	85
E18 rat septum	800–870	4	76	77
E18 rat corpus striatum	980–1,160	4	73	76

* This represents the number of neurons surviving per well after 24 h of culture. The number of cells originally seeded was 2,000 for each of the PNS sources (40–50% of which were neurons) and 1,000 cells for E4 cord. The number of cells seeded per well for the other CNS sources was 2,000 but the proportion of neurons present in these dissociates was not determined.

plants on plastic substrata treated with 40 μg/ml fibronectin. Akers et al. (18) have reported that fibronectin, bound to plastic or polylysine substrata, elicits neurite outgrowth from reaggregate cultures of chick embryo retinal cells. They further report that less fibronectin-induced neurite outgrowth occurs when fibronectin is bound to plastic at pH 7 than at pH 6. However, even in optimal conditions, half-maximal neurite growth effects required 10 μg/ml of fibronectin during substratum pretreatment. No attempt was made in this study to examine the pH dependency of fibronectin- or laminin-induced neurite promotion, but our results obtained with fibronectin coating at pH 7 were similar to Akers et al. (18).

PNPF have been repeatedly observed in glial, heart, and other cell-conditioned media (8–10, 13, 14,17,18). PNPF have been suggested to be glycoproteins like laminin and fibronectin, or proteoglycans (17, 36, 49). Like laminin, PNPF are effective on several ganglionic (14, 15), spinal cord (15, 16), and retinal (17) neurons in monolayer cultures. Nevertheless, PNPF-1–induced neurite-promoting activity is not blocked by laminin antiserum (Fig. 3) and thus constitutes a distinct category of substratum-binding neurite-promoting agents. Lander et al. (49) have reported that conditioned medium from bovine corneal endothelial cells contains a polylysine-binding neurite-promoting factor for sympathetic neurons that apparently requires heparan sulfate proteoglycan for its activity. We tested a heparan sulfate proteoglycan purified from a rat hepatoma (25) as well as a chondroitin sulfate proteoglycan from a rat yolk sac tumor (24) for neurite-promoting activity and found them both to be inactive. It remains to be determined whether the proteoglycan studied by Lander et al. (49) does have inherent neurite-promoting activity or whether it serves as a carrier for a separate neurite-promoting agent, which itself may or may not be identical to laminin or PNPF-1.

The potent activity of laminin and PNPF-1 as neurite-promoting factors in vitro has considerable implications in vivo. Laminin is a constituent of basal lamina, the ECM structure observed at the interfaces between mesodermal and ectodermal tissues (42, 50). Basal lamina is particularly abundant in peripheral nerve (51), but is restricted to perivascular spaces and meninges in central nervous tissue (52). Laminin (and PNPF) can be produced and secreted by Schwann cells (13, 53, 54) and PNPF is produced by CNS glia in culture (15). Neuroblastoma cells, a PNS-derived neuronal line, have also been reported to produce laminin in vitro and to deposit laminin on the substratum occupied by their neuritic extensions (55). Axonal regeneration occurs readily in peripheral nerve, and the properties and organization of its Schwann cells may be important contributors to axonal regrowth (56). In contrast, in the adult mammal, axons in central nervous tissue usually fail to regenerate (1, 2). Firm evidence has been recently produced that central axons will regenerate as readily as peripheral ones if provided with peripheral nerve as the regeneration microenvironment (3, 4, 57). It is an attractive speculation, therefore, that the restricted availability of laminin (and/or PNPF) may be important in the regulation of axonal regeneration in the CNS in vivo. Newly developed culture systems for central neurons (30, 34) and the availability of new models for in vivo regeneration studies (58–60) should make it possible to submit this speculation to experimental testing.

We thank Drs. Michael Brennan and Ake Oldberg for the proteoglycan samples.

This work was supported by grant NS-16349 (SV) from the National Institute for Neurological and Communicative Diseases and Stroke and grant AM 30051 (EE) from the National Institute of Arthritis and Metabolic Diseases and grant CA 28896 (ER) and Cancer Center Support Grant CA 30199 from the National Cancer Institute, Dept. of Health and Human Services.

Received for publication 29 March 1983, and in revised form 8 July 1983.

REFERENCES

1. Guth, L. 1956. Regeneration in the mammalian peripheral nervous system. *Physiol. Rev.* 36:441–478.
2. Clemente, C. D.1964. Regeneration in the vertebrate central nervous system. *Int. Rev. Neurobiol.* 6:257–301.
3. Benfey, M., and A. J. Aguayo. 1982. Extensive elongation of axons from rat brain into peripheral nerve grafts. *Nature (Lond.)* 296:150–152.
4. Aguayo, A. J., S. David, P. Richardson, and G. Bray. 1982. Axonal elongation in peripheral and central nervous system transplants. *Adv. Cell. Neurobiol.* 3:215–234.
5. Varon, S., and R. Adler. 1981. Trophic and specifying factors directed to neuronal cells. *Adv. Cell. Neurobiol.* 2:115–163.
6. Varon, S., and R. Bunge. 1978. Trophic mechanisms in the peripheral nervous system. *Annu. Rev. Neurosci.* 1:327–361.
7. Letourneau, P. C. 1975. Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. *Dev. Biol.* 44:77–91.
8. Hawrot, E. 1980. Cultured sympathetic neurons: effects of cell-derived and synthetic substrata on survival and development. *Dev. Biol.* 74:136–151.
9. Collins, F. 1978. Induction of neurite outgrowth by conditioned medium factor bound to the culture substratum. *Proc. Natl. Acad. Sci. USA.* 75:5210–5213.
10. Dribin, L. B., and J. N. Barrett. 1980. Conditioned medium enhances neurite outgrowth from rat spinal cord explants. *Dev. Biol.* 74:184–195.
11. Kligman, D. 1982. Isolation of a protein from bovine brain which promotes neurite extension from chick embryo cerebral cortex neurons in defined medium. *Brain Res.* 250:93–100.
12. Henderson, C. E., M. Huchet, and J. P. Changeux. 1981. Neurite outgrowth from embryonic chicken spinal neurons is promoted by media conditioned by muscle cells.

Proc. Natl. Acad. Sci. USA. 78:2625–2629.
13. Manthorpe, M., S. Varon, and R. Adler. 1981. Neurite-promoting factor (NPF) in conditioned medium from RN22 Schwannoma cultures: bioassay, fractionation and other properties. *J. Neurochem.* 37:759–767.
14. Adler, R., and S. Varon. 1979. Cholinergic neuronotrophic factors. V. Segregation of survival- and neurite-promoting activities in heart conditioned media. *Brain Res.* 188:437–448.
15. Adler, R., M. Manthorpe, S. D. Skaper, and S. Varon. 1981. Polyornithine-attached neurite promoting factors (PNPF's): culture sources and responsive neurons. *Brain Res.* 206:129–144.
16. Longo, F. M., M. Manthorpe, and S. Varon. 1982. Spinal cord neuronotrophic factors (SCNTFs). I. Bioassay of Schwannoma and other conditioned media. *Dev. Brain Res.* 3:277–294.
17. Adler, R. 1982. Regulation of neurite growth in purified retina neuronal cultures: effects of PNPF, a substratum-bound neurite-promoting factor. *J. Neurosci. Res.* 8:165–178.
18. Akers, R. M., D. F. Mosher, and J. E. Lilien. 1981. Promotion of retinal neurite outgrowth by substratum-bound fibronectin. *Dev. Biol.* 86:179–188.
19. Timpl, R., H. Rohde, L. Risteli, U. Ott, P. Gehron-Robey, and G. R. Martin. 1980. Laminin. *Methods Enzymol.* 82:831–838.
20. Baron van Evercooren, A., H. K. Kleinman, S. Ohno, P. Marangos, J. P. Schwartz, and M. E. Dubois-Dalcq. 1982. Nerve Growth Factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* 8:179–194.
21. Skaper, S. D., R. Adler, and S. Varon. 1979. A procedure for purifying neuron-like cells in cultures from central nervous tissues with a defined medium. *Dev. Neurosci.* 2:233–237.
22. Engvall, E., T. Krusius, U. Wewer, and E. Ruoslahti. 1983. Laminin from rat yolk sac tumor: isolation, partial characterization, and comparison with mouse laminin. *Arch. Biochem. Biophys.* 222:649–656.
23. Ruoslahti, E., E. Hayman, M. Pierschbacher, and E. Engvall. 1980. Fibronectin: purification, immunochemical properties and biological activities. *Methods Enzymol.* 82A:803–831.
24. Oldberg, A., E. G. Hayman, and E. Ruoslahti. 1981. Isolation of a chondroitin sulfate proteoglycan from a rat yolk sac tumor and immunochemical demonstration of its cell surface localization. *J. Biol. Chem.* 256:10847–10852.
25. Oldberg, A., C. Schwartz, and E. Ruoslahti. 1982. Isolation and partial characterization of a rat hepatoma heparan sulfate proteoglycan. *Arch. Biochem. Biophys.* 216:400–406.
26. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
27. Varon, S., M. Manthorpe, and R. Adler. 1979. Cholinergic neuronotrophic factors. I. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. *Brain Res.* 173:24–45.
28. Varon, S., S. D. Skaper and M. Manthorpe. 1981. Tropic activities for dorsal root and sympathetic ganglionic neurons in media conditioned by Schwann and other peripheral cells. *Dev. Brain Res.* 1:73–87.
29. Adler, R., M. Manthorpe, and S. Varon. 1979. Separation of neuronal and non-neuronal cells in monolayer cultures from chick embryo optic lobe. *Dev. Biol.* 69:424–435.
30. Barbin, B., I. Selak, M. Manthorpe, and S. Varon. 1983. Use of central neuronal cultures for the detection of neuronotrophic agents. *Neurosci.* In press.
31. Manthorpe, M., S. D. Skaper, R. Adler, K. B. Landa, and S. Varon. 1980. Cholinergic neuronotrophic factors. IV. Fractionation properties of an extract from selected chick embryonic eye tissues. *J. Neurochem.* 34:69–75.
32. Varon, S., J. Nomura, J. R. Perez-Polo, and E. M. Shooter. 1972. The isolation and assay of the Nerve Growth Factor proteins. *In* Methods and Techniques of Neurosciences. R. Fried, editor. Marcel Dekker, Inc., New York. 203–209.
33. Manthorpe, M., F. M. Longo, and S. Varon. 1982. Comparative features of spinal neuronotrophic factors in fluids collected in vitro and in vivo. *J. Neurosci. Res.* 8:241–250.
34. Varon, S., S. D. Skaper, G. Barbin, I. Selak, and M. Manthorpe. 1983. Low molecular weight agents support the survival of cultured neurons from the central nervous system. *J. Neurosci.* In press.
35. Manthorpe, M., S. D. Skaper, G. Barbin, and S. Varon. 1982. Cholinergic neuronotrophic factors (CNTF's). VII. Concurrent activities on certain Nerve Growth Factor-responsive neurons. *J. Neurochem.* 38:415–421.
36. Adler, R., M. Manthorpe, and S. Varon. 1983. Lectin reactivity of PNPF, a polyornithine-binding neurite promoting factor. *Dev. Brain Res.* 6:69–75.
37. Manthorpe, M., S. D. Skaper, and S. Varon. 1981. Neuronotrophic factors and their antibodies: in vitro microassays for titration and screening. *Brain Res.* 230:295–306.
38. Engvall, E., M. L. Bell, R. N. K. Carlsson, E. J. Miller, and E. Ruoslahti. 1982. Non-helical fibronectin-binding basement membrane collagen from endodermal cell cultures. *Cell.* 29:475–482.
39. Manthorpe, M., R. Adler, and S. Varon. 1979. Development, reactivity and GFA immunofluorescence of astroglia-containing monolayer cultures from rat cerebrum. *J. Neurocytol.* 8:605–621.
40. McCarthy, K., and J. de Vellis. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissues. *J. Cell Biol.* 85:890–902.
41. Manthorpe, M., S. D. Skaper, and S. Varon. 1980. Purification of mouse Schwann cells using neurite-induced proliferation in serum-free monolayer cultures. *Brain Res.* 196:467–486.
42. Timpl, R., H. Rohde, P. G. Roley, S. I. Rennard, J. M. Foidard, and G. R. Martin. 1979. Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933–9937.
43. Carlsson, R., E. Engvall, A. Freeman, and E. Ruoslahti. 1981. Laminin and fibronectin in cell adhesion of cells from regenerating liver to laminin. *Proc. Natl. Acad. Sci. USA.* 78:2403–2406.
44. Vlodavsky, I., and D. Gospodarowicz. 1981. Respective roles of laminin and fibronectin in adhesion of human carcinoma and sarcoma cells. *Nature (Lond.)* 289:304–306.
45. Terranova, V. P., C. N. Rao, T. Kalebic, I. M. Margulies, and L. A. Liotta. 1983. Laminin receptor on human breast carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:444–448.
46. Couchman, J. R., M. Hook, D. A. Rees, and R. Timpl. 1983. Adhesion, growth, and matrix production by fibroblasts on laminin substrates. *J. Cell Biol.* 96:177–183.
47. Grover, A., G. Andrews, and E. D. Adamson. 1983. Role of laminin in epithelium formation by F9 aggregates. *J. Cell Biol.* 97:137–144.
48. Wewer, U., R. Albrechtsen, M. Manthorpe, S. Varon, E. Engvall, and E. Ruoslahti. 1983. Biologically active, nearly intact human laminin isolated from placenta. *J. Biol. Chem.* In press.
49. Lander, A. D., D. K. Fujii, D. Gospodarowicz, and L. F. Reichardt. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. *J. Cell Biol.* 94:574–585.
50. Kefalides, N. A., ed. 1978. Biology and Chemistry of Basement Membranes. Academic Press, New York.
51. Thomas, P. K. 1966. The cellular response to nerve injury. I. The cellular outgrowth from the distal stump of transected nerve. *J. Anat.* 100:287–303.
52. Shellswell, G.B., D. J. Restall, V. C. Duance, and A. J. Bailey. 1979. Identification and differential distribution of collagen types in the central and peripheral nervous systems. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 106:305–308.
53. Roufa, D., C. Cornbrooks, M. Johnson, and M. Bunge. 1982. Preparation of homogeneous populations of immature Schwann cells from early embryonic sympathetic ganglia. *Soc. Neurosci. Abstr.* 8:240.
54. Cornbrooks, C., D. Carey, R. Timpl, J. McDonald, and R. Bunge. 1982. Immunohistochemical visualization of fibronectin and laminin in adult rat peripheral nerve and peripheral nerve cells in culture. *Soc. Neurosci. Abstr.* 8:240.
55. Alitalo, K., M. Kurkinen, I. Virtanen, K. Mellström, and A. Vaheri. 1982. Deposition of basement membrane proteins in attachment and neurite formation of cultured murine C-1300 neuroblastoma cells. *J. Cell. Biochem.* 18:25–35.
56. Thomas, D. K. 1974. Nerve Injury. *In* Essays on the Nervous System. R. Bellairs and E. G. Gray, editors. Oxford University Press (Clarendon), London and New York. pp. 44–70.
57. David, S., and A. J. Aguayo. 1981. Axonal elongation into peripheral nervous system bridges after central nervous system injury. *Science (Wash. DC).* 214:931–933.
58. Varon, S., and G. Lundborg 1982. In vivo model for peripheral nerve regeneration and the presence of neuronotophic factors. *In* Nervous System Regeneration. A. M. S. Giuffrida, B. Haber, G. Hashim, and R. Perez-Polo, editors. Alan R. Liss, Inc., New York. 221–240.
59. Nieto-Sampedro, M., E. R. Lewis, C. W. Cotman, M. Manthorpe, S. D. Skaper, G. Barbin, F M. Longo, and S. Varon. 1982. Brain injury causes a time-dependent increase in neuronotrophic activity at the lesion site. *Science (Wash. DC).* 217:860–861.
60. Manthorpe, M., M. Nieto-Sampedro, S. D. Skaper, E. R. Lewis, G. Barbin, F. M. Longo, C. W. Cotman, and S. Varon. 1983. Neuronotrophic activity in brain wounds in the developing rat. Correlation with implant survival in the wound cavity. *Brain Res.* 267:47–56.