Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8726–8729, December 1987
Neurobiology

Solubilization of a membrane factor that stimulates levels of substance P and choline acetyltransferase in sympathetic neurons

(neuronotrophic factor/plasma membrane/cell–cell contact)

VIVIEN WONG* AND JOHN A. KESSLER

Departments of Neurology and Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461

Communicated by Michael V. L. Bennett, August 19, 1987

ABSTRACT **The choice of which neurotransmitters will be produced by a developing neuron is influenced by the microenvironment of the neuron. In this study we show that neuronal contact with membrane-associated molecules promotes expression of peptidergic and cholinergic traits. Treatment of cultured neonatal rat sympathetic neurons with plasma membranes derived from adult rat spinal cord or sympathetic ganglia induced expression of the peptide transmitter substance P and increased levels of the cholinergic biosynthetic enzyme choline acetyltransferase. The transmitter-stimulating activity could be solubilized from spinal cord membranes by the detergent octyl glucoside but not by Triton X-100. The choline acetyltransferase- and substance P-stimulating activity also could be extracted from spinal cord membranes by 4 M sodium chloride, suggesting that the active material is membrane associated rather than an intrinsic structural membrane molecule. Trypsin or heat treatment of the extract destroyed the transmitter-stimulating activity, indicating that the factor contains a protein. Activity also was destroyed by hyaluronidase treatment, suggesting that the active material may contain a glycosaminoglycan. The choline acetyltransferase-stimulating activity in the 4 M NaCl extract was eluted in a single peak from a calibrated Sephadex G-75 column with a retention time slightly less than that of a 25-kDa standard. $NaDodSO_4$/polyacrylamide gel electrophoresis of the active peak revealed a predominant band at 29 kDa. Thus, contact-mediated stimulation of substance P and choline acetyltransferase activity in sympathetic neurons results from neuronal exposure to a 29-kDa membrane-associated factor.**

Neuronal contact with other cell membranes plays an important role in regulating growth and development of the nervous system. For example, cell contact appears to regulate neuronal morphogenesis, to guide outgrowth of neurites, and to facilitate recognition of appropriate target cells (1–6). However, the role of cell–cell contact in neuronal differentiation and neurotransmitter expression is less clearly understood. Culture of sympathetic neurons on a substrate of fixed heart cells resulted in an increase of cholinergic traits (7). Similarly, the development of mesencephalic dopaminergic neurons was stimulated by contact with striatal cell membranes (8). Plasma membranes prepared from neurons and Schwann cells strikingly increased choline acetyltransferase (ChoAcTase) activity and induced substance P (SP) expression in pure sympathetic neuron cultures (9). Furthermore, membranes isolated from adrenal chromaffin cells greatly increased tyrosine hydroxylase (TyrOHase) levels in the same cell type *in vitro* (10). These observations suggest that neuronal interactions with cell-surface and matrix molecules may play a decisive role in neuronal differentiation and transmitter expression. However, the molecules mediating these interactions have not been identified fully.

The present study was undertaken to characterize cell surface molecules that influence cholinergic and peptidergic transmitter expression during development of sympathetic neurons. We find that membranes prepared from rat spinal cord contain a 29-kDa cholinergic and peptidergic promoting factor that can be successfully solubilized by detergent or extracted by high-salt-containing buffer.

METHODS

Preparation, Solubilization, and Extraction of Crude Membranes. Entire spinal cords or superior cervical ganglia (SCG) were dissected from female Sprague–Dawley rats (175–225 g), rinsed with ice-cold physiologic saline, and weighed. All steps were done at 4°C. Tissues were suspended in 10 vol of 0.2 M Tris buffer (pH 7.4) containing 0.32 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 50 μg of bacitracin per ml, and 10 mM ethylenediaminetetraacetic acid and were homogenized with a Polytron for 15 sec at medium speed. Cell debris and nuclei were removed by centrifugation at 600 × *g* for 10 min, and the supernatant was then centrifuged at 100,000 × *g* for 1 hr. The resulting pellet was resuspended in (*i*) 100 mM octyl glucoside, (*ii*) 50 mM octyl glucoside, (*iii*) 4 M NaCl, (*iv*) 0.1% Triton-X 100, or (*v*) buffer and was allowed to sit for 1 hr at 4°C with intermittent agitation. The suspension was then centrifuged at 100,000 × *g* for 1 hr, and both supernatant and pellet were collected for testing for biological activity. Ten micrograms of each preparation was tested per culture (1.5 ml), with final solvent concentrations of 0.3 mM octyl glucoside, 26 mM NaCl, or 0.00066% Triton.

Treatments of Salt Extract of Membrane. The extract was incubated with (*i*) trypsin (GIBCO) at 2 mg/ml, (*ii*) heparinase (Sigma; 100 units per mg) at 2 units per ml, (*iii*) testicular hyaluronidase (Sigma, type IV S; 1000 units per mg) at 2 mg/ml, or (*iv*) buffer for 4 hr at 37°C. All treatments except for trypsin were done in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, bacitracin at 50 μg/ml, and 10 mM EDTA). Heat inactivation was carried out at 90°C for 10 min. Equal amounts of 4 M NaCl in buffer were added to all control dishes.

Purification of the Salt Extract. To purify the factor, 1 ml of the salt extract was filtered twice through a Sephadex G-75 ultrafine column at a linear flow rate of 5 cm/hr and was eluted with buffer containing 20 mM Tris·HCl and 300 mM NaCl (pH 7.7). The resulting peaks were tested for ChoAcTase-stimulating activity in SCG cultures and were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ChoAcTase, choline acetyltransferase; SP, substance P; TyrOHase, tyrosine hydroxylase; MANS, membrane-associated neurotransmitter stimulating factor; SCG, superior cervical ganglion/ganglia.

*To whom reprint requests should be addressed at: Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

examined by using $NaDodSO_4$/10% polyacrylamide gel electrophoresis (11) with visualization by Coomassie blue stain.

Tissue Culture Techniques. The neonatal SCG was dissociated and grown as described (12) in a medium consisting of Ham's nutrient mixture F-12 (GIBCO) with 10% fetal calf serum (HyClone, Logan, UT), nerve growth factor β (100 ng/ml), penicillin (GIBCO) (50 units per ml), and streptomycin (GIBCO) (50 μg/ml). Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere at nearly 100% relative humidity. Cultures were fed three times per week (Monday, Wednesday, Friday) in all experiments. Ganglion nonneuronal cells were eliminated by treatment on days 1 and 3 of culture with cytosine arabinonucleoside (5 μM). Virtually 100% of the remaining cells (8,000–11,000 per dish) bound tetanus toxin and had the morphology of neurons (13). Cell numbers were determined by counting as described (13).

Biochemical Assays. Cultures were harvested for measurment of SP, ChoAcTase, and TyrOHase as described (13). ChoAcTase was assayed by using minor modifications of the method of Fonnum (14). SP was measured by radioimmunoassay as reported previously with antisera generated and characterized in our laboratory (13). TyrOHase was measured as described (12). Nerve growth factor β was prepared by the method of Mobley *et al.* (15).

Statistics. Data were analyzed by Student's t-test and analysis of variance when appropriate.

RESULTS

Effects of Crude Membrane Preparation. Sympathetic neurons (8,000–11,000 neurons per dish) were treated with crude membranes prepared from the rat SCG (8 μg/ml) or spinal cord (50 μg/ml). After 7 days of treatment, the cultures were examined for content of ChoAcTase and SP (Fig. 1). Membranes from both sources significantly elevated ChoAcTase activity from 0.7 to 3.4 fmol per neuron per min (spinal cord membranes) or 5.1 fmol per neuron per min (SCG membranes). SP, which normally is not expressed in sympathetic neurons cultured at this density (13), was induced to a significant level of 6.2 fg per neuron by spinal cord membranes and 2.4 fg per neuron by SCG membranes.

Effects of Solubilized Spinal Cord Membranes. When spinal cord membranes were solubilized with 100 mM octyl glucoside, the resulting supernatant (1.5–2.0 mg of protein per ml) stimulated ChoAcTase activity 5.6-fold in SCG neurons (Fig. 2). SP was also induced dramatically to 21.3 fg per neuron. Pelleted material remaining after octyl glucoside treatment still retained some ChoAcTase-promoting properties (data not shown), suggesting that solubilization was incomplete. Treatment of spinal cord membranes with 50 mM octyl glucoside or 0.1% Triton X-100 (Fig. 3) did not solubilize the ChoAcTase-stimulating molecules. Treatment of control cultures with the above detergents had no effect.

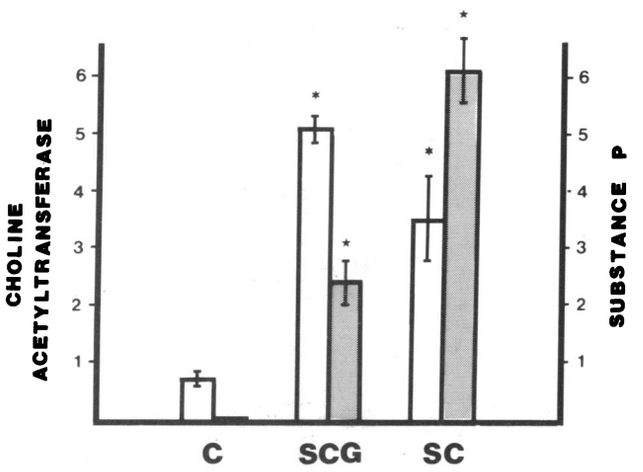

FIG. 1. Effects of isolated membranes on ChoAcTase activity and SP levels in sympathetic neurons. Cultures of sympathetic neurons were treated on days 3, 5, and 7 with spinal cord (SC) membranes at 50 μg/ml or SCG membranes at 8 μg/ml. Cultures were harvested on day 9 and were assayed for ChoAcTase activity (open bars) or SP (shaded bars). ChoAcTase is expressed in fmol per neuron per min (mean ± SEM); SP is expressed in fg per neuron (mean ± SEM) (n = 3). *Differs from control (C) at $P < 0.002$.

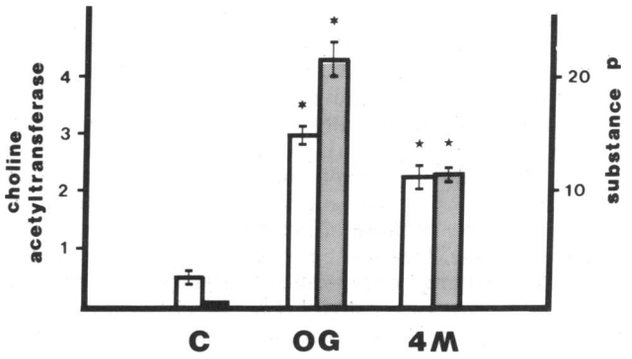

FIG. 2. Spinal cord membranes were extracted with either 4 M NaCl (4 M) or 100 mM octyl glucoside (OG). Sympathetic neuron cultures were treated on days 3, 5, and 7 with the 4 M extract (6.5 μg/ml) or the OG extract (20 μg/ml). On day 9 the cultures were harvested and assayed for ChoAcTase activity (open bars) and SP (shaded bars). Values are expressed as in Fig. 1; n = 3. *Differs from control (C) at $P < 0.001$.

Effects of Salt Extract of Spinal Cord Membranes. When spinal cord membranes were treated with 4 M NaCl, the resulting supernatant (0.5–0.65 mg of protein per ml) contained the ChoAcTase-stimulating and SP-inducing activity. Treatment of sympathetic neurons with this extract increased ChoAcTase activity 4-fold (Figs. 2 and 3) and induced SP from undetectable levels to 11.2 fg per neuron (Fig. 2). Neuron numbers were unchanged by the treatment, and variations in cell density within the range utilized (8,000–11,000 neurons per dish) did not alter neuronal responses to treatments. The ability of high-salt buffers to extract activity suggested that some or all of the active material was membrane associated instead of being an integral constituent of the membranes; 2 M NaCl was less effective in extracting this

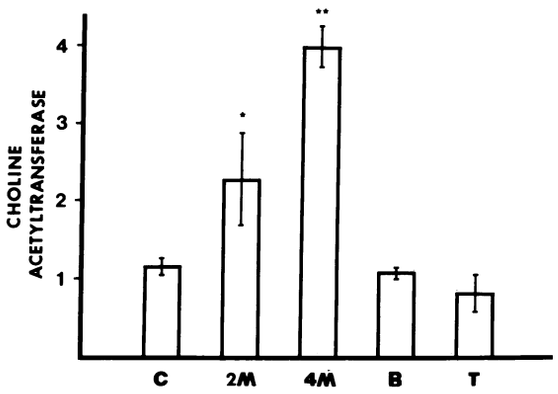

FIG. 3. Extraction of spinal cord membranes with NaCl. Cultures of sympathetic neurons were treated with the 4 M NaCl extract (4 M) of spinal cord membranes at 5.0 μg/ml on days 3, 5, and 7 of culture. On day 9 the cultures were harvested and assayed for ChoAcTase activity. Extraction by 2 M NaCl (2 M), buffer (B), and Triton X-100 (T) had less or no effect. Values are expressed as in Fig. 1; n = 3. *Differs from control at $P < 0.02$. **Differs from control (C) at $P < 0.005$.

membrane-associated neurotransmitter-stimulating factor (MANS), and control buffer did not extract any transmitter-stimulating activity (Fig. 3). Treatment of control cultures with 4 M NaCl alone did not have any effect (Fig. 3). TyrOHase in the same cultures was slightly decreased or unchanged (data not shown), indicating the specificity of the factor. The remaining membranes after salt extraction still retained some ability to stimulate ChoAcTase (data not shown), possibly because of incomplete extraction.

Dose Response. Treatment of sympathetic neurons with the MANS extract at a concentration of 800 ng/ml significantly increased ChoAcTase activity (Fig. 4). Increasing the dose resulted in a proportional increase in ChoAcTase activity up to a dose of 3.25 μg/ml. However, further increase of the dose from 3.25 to 13.00 μg/ml did not further increase ChoAcTase activity, suggesting that saturation was achieved at 3.25 μg/ml.

Characterization of MANS. Trypsin treatment or heating MANS to 90°C destroyed the stimulating activity, suggesting that MANS included a protein (Fig. 5). Testicular hyaluronidase treatment (in the presence of protease inhibitors) also destroyed activity, suggesting that a glycosaminoglycan was part of MANS. The ChoAcTase-stimulatory activity was not sensitive to heparinase treatment, and it was largely stable at 37°C for 4 hr (Fig. 5).

Purification of MANS. The ChoAcTase-stimulating activity was eluted in a single peak from a calibrated Sephadex G-75 column with a retention time slightly less than that of a 25-kDa standard (chymotrypsinogen A). $NaDodSO_4$/polyacrylamide gel electrophoresis of the active peak (Fig. 6) revealed a predominant band at 29 kDa. Overloading a gel with excess protein revealed a few additional faint bands.

DISCUSSION

Previous studies have shown that altered cell density in culture may profoundly influence neurotransmitter expression. Levels of ChoAcTase activity were stimulated by increasing cell density in PC12 (16, 17), striatal (18), septal (19), and sympathetic neuronal (13, 20) cultures, and TyrOHase was increased in high-density cultures of adrenal chromaffin cells (10, 21). These changes apparently were mediated by cell-surface molecules rather than soluble diffusible factors because treatments with isolated plasma membranes or culture on layers of aldehyde-fixed cells reproduced the effects. Moreover, plasma membranes isolated either from sympathetic neurons or from their associated nonneuronal cells (principally Schwann cells) stimulated ChoAcTase activity and SP in SCG neurons in culture (9). To further characterize and purify the component(s) mediating these effects, a more abundant membrane source was essential. After studying numerous tissues (unpublished data), we chose the rat spinal cord as the membrane source because of its effectiveness, abundance, and ready availability. The spinal cord is a heterogeneous tissue with many types of

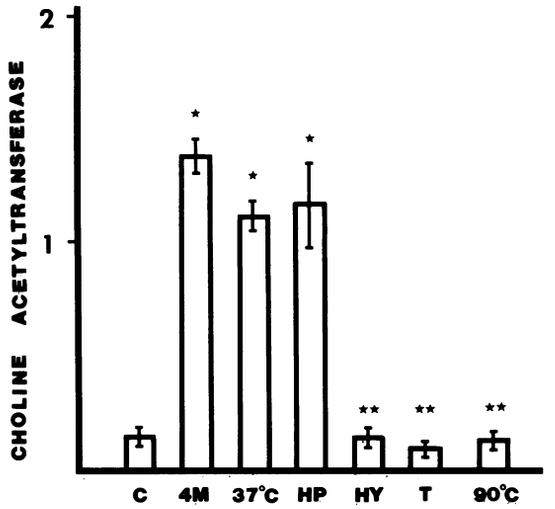

FIG. 5. Characterization of the active factor in the 4 M NaCl extract. Aliquots of the 4 M NaCl (4 M) extract were treated with enzymes (heparinase, HP; testicular hyaluronidase, HY; and trypsin, T) or were heated (37°C and 90°C), and the treated aliquots were used to treat sympathetic neurons on days 3, 5, and 7 of culture. On day 9 the cultures were harvested and assayed for ChoAcTase activity. Values are expressed as in Fig. 1; $n = 3$. *Differs from control (C) at $P < 0.005$. **Differs from 37°C heated extract at $P < 0.002$.

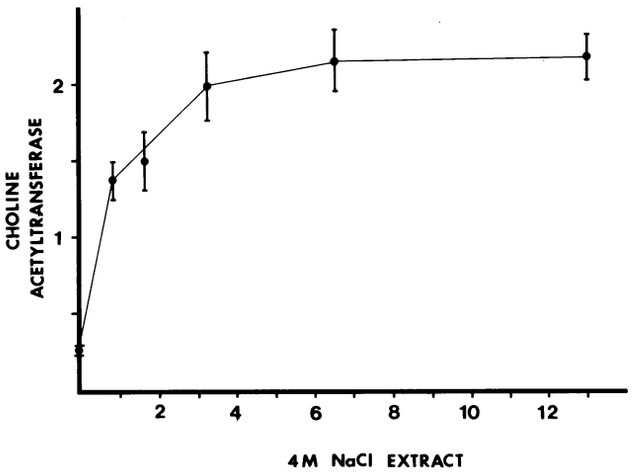

FIG. 4. Effects of increasing doses of the 4 M NaCl extract on ChoAcTase activity. Sympathetic neuron cultures were treated on days 3, 5, and 7 of culture with different doses (μg/ml) of the 4 M NaCl extract. On day 9 the cultures were harvested for measurement of ChoAcTase activity; $n = 3$ for each dose.

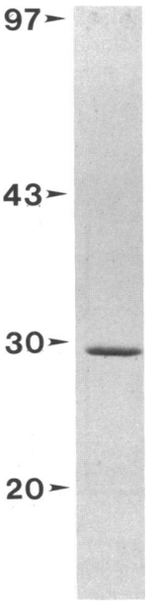

FIG. 6. Apparent molecular mass of the ChoAcTase-stimulating factor. MANS was passed through a Sephadex G-75 column twice. The fraction that contained the ChoAcTase-stimulating property was electrophoresed on $NaDodSO_4$/10% polyacrylamide gel, and the bands were visualized by Coomassie blue stain.

neurons and nonneuronal cells, and we do not yet know which cell type(s) contributed MANS. However, it is hoped that development of a specific antibody will facilitate immunocytochemical localization in the spinal cord and elsewhere.

The ChoAcTase-stimulating activity was eluted from a Sephadex G-75 column in a single peak; $NaDodSO_4$/polyacrylamide gel electrophoresis of the active peak revealed a single predominant band at 29 kDa. However, it has not yet been conclusively established that the 29-kDa molecule is the active factor, since it is possible that the ChoAcTase-stimulating activity is due to a minor contaminant.

Although the active material is membrane associated, it is possible that under certain conditions it may be shed or sloughed off and, thus, occur in a solubilized form. However, this molecule is clearly different from other well-studied soluble ChoAcTase-stimulating factors found in heart cell-, fibroblast-, and pineal cell-conditioned media (22–25) and in brain (9). First, the membrane factor induced the expression of SP in pure sympathetic neuronal cultures, whereas other soluble ChoAcTase-stimulating factors do not (22, 26). Moreover, simultaneous treatment of sympathetic neurons with saturating doses of the membrane factor and with medium conditioned by fibroblasts resulted in further increases in ChoAcTase activity (26), indicating that the membrane factor and the soluble factors interacted at different sites on the SCG membranes. Finally, the apparent molecular weight of the membrane factor of 29 kDa is smaller than the 45- to 55-kDa size of the soluble factors (22, 24, 25).

The transmitter-stimulating activity was destroyed by both trypsin and by heat, indicating that the active material has a protein domain. Activity also was destroyed by testicular hyaluronidase, which digests any of several glycosaminoglycans by hydrolyzing the β or α 1→4 linkage between galactosamine or glucosamine and glucuronic acid (27). Although this observation suggests that the factor includes a glycosaminoglycan, interpretation is complicated because hyaluronidase often is contaminated with proteases. Even though protease inhibitors were included with the hyaluronidase treatment, further studies will be necessary to ascertain whether the factor does, in fact, contain a glycosaminoglycan.

The initial expression of ChoAcTase and other cholinergic traits by neural crest cells occurs extremely early in gestation (28), coinciding with the neural crest cell aggregation that occurs shortly before migration commences (29). Our observations regarding the cholinergic promoting actions of a membrane-associated factor suggest that the process of crest-cell aggregation may lead to this early expression of cholinergic traits. The early expression of ChoAcTase and other cholinergic traits appears to be transient for many crest cells, disappearing as the cells migrate. It is tempting to speculate that this loss of cholinergic traits reflects changes in cell-surface molecules and/or extracellular matrix to which the migrating crest cells are exposed. By extension, it is possible that other well-documented examples of transient expression of neurotransmitters (30, 31) may reflect changes in the surrounding cellular and extracellular matrix molecules.

We thank Ms. Mary Jane Dougherty and Mrs. Antoinette Barnecott for their excellent technical assistance. This work was supported by grants from the National Institutes of Health (NS 20013 and NS 20778) and the United Cerebral Palsy Association.

1. Edelman, G. M. (1984) *Annu. Rev. Neurosci.* **7,** 339–377.
2. Bunge, R., Gluser, L., Liebermann, M., Ruben, D., Salzer, J., Whittenberger, B. & Woolsey, H. (1970) *J. Supramol. Struct.* **11,** 175–187.
3. Goodman, C. S. (1982) in *Neuronal Development*, ed. Spitzer, N. C. (Plenum, New York), pp. 171–212.
4. Rutishauser, U. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **48,** 501–514.
5. Keshishian, H. & Bentley, D. (1983) *Dev. Biol.* **96,** 89–102.
6. Frazier, W. & Glaser, L. (1979) *Annu. Rev. Biochem.* **48,** 491–523.
7. Hawrot, E. (1980) *Dev. Biol.* **74,** 136–151.
8. Prochiantz, A., Dagnet, M.-C., Herbert, A. & Glowinski, J. (1981) *Nature (London)* **293,** 570–572.
9. Kessler, J. A., Conn, G. & Hatcher, V. B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3528–3532.
10. Saadat, S. & Thoenen, H. (1986) *J. Cell Biol.* **103,** 1991–1997.
11. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
12. Kessler, J. A. (1984) *Dev. Biol.* **106,** 61–69.
13. Kessler, J. A. (1985) *Neuroscience* **15,** 827–839.
14. Fonnum, F. J. (1969) *Biochem. J.* **115,** 465–472.
15. Mobley, W. C., Schenker, A. & Shooter, E. M. (1976) *Biochemistry* **15,** 5543–5551.
16. Greene, L. A. & Rein, G. (1977) *Nature (London)* **268,** 349–351.
17. Edgar, D. H. & Thoenen, H. (1978) *Brain Res.* **154,** 186–190.
18. Kessler, J. A. (1986) *Dev. Biol.* **113,** 77–89.
19. Hefti, F., Hartikka, J., Eckenstein, F., Gnahn, H., Heumann, R. & Schwab, M. (1985) *Neuroscience* **14,** 55–68.
20. Adler, J. & Black, I. B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4296–4300.
21. Acheson, A. L. & Thoenen, H. (1983) *J. Cell Biol.* **97,** 925–928.
22. Kessler, J. A. (1984) *Brain Res.* **321,** 155–159.
23. Patterson, P. H. & Chun, L. L. Y. (1977) *Dev. Biol.* **56,** 263–280.
24. Fukada, K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8795–8799.
25. Weber, M. (1981) *J. Biol. Chem.* **256,** 3447–3453.
26. Kremer, N., Wong, V. & Kessler, J. A. (1987) *Soc. Neurosci. Abstr.* **13,** 924.
27. Hay, E. (1981) *Cell Biology of Extracellular Matrix* (Plenum, New York).
28. LeDouarin, N. M. (1980) *Nature (London)* **286,** 663–668.
29. Tosney, K. W. (1978) *Dev. Biol.* **62,** 317–330.
30. Black, I. B. (1982) *Science* **215,** 1198–1204.
31. Gershon, M. D., Rothman, T. P., Joh, T. H. & Teitelman, G. (1984) *J. Neurosci.* **4,** 2269–2280.