Localized Survival of Ciliary Ganglionic Neurons Identifies Neuronotrophic Factor Bands on Nitrocellulose Blots¹

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Abstract

A novel and sensitive method has been developed to identify ciliary neuronotrophic factors (CNTFs) from tissue extracts after blotting to nitrocellulose paper. The CNTF proteins are required for the *in vitro* survival of embryonic chick ciliary ganglionic neurons. Tissue extracts containing such CNTFs are electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Purified ciliary ganglionic neurons are seeded on the surface of the nitrocellulose blot, and the culture is incubated for 24 hr in medium lacking CNTF. CNTF can be localized on the blot because it retains its ability to support the survival of the neurons cultured on the nitrocellulose. A band of viable neurons, easily visualized by staining with a vital dye, is supported by the blotted CNTF polypeptide. The number of neurons surviving on the blotted CNTF is related to the amount of CNTF originally loaded on the electrophoretic gel. As little as 2 ng (16 trophic units) of CNTF protein contained in crude tissue extracts can be loaded on the sodium dodecyl sulfate gel and still be recognized by the cultured neurons. This method was used to identify CNTF polypeptides from extracts of adult rat nerve (24,000 and 19,000 daltons) and from tissue found near experimentally induced adult rat brain lesions (24,000 daltons). The electrophoretic mobilities of these peptides are distinct from the previously purified chick eye CNTF polypeptide (20,400 daltons).

Neuronotrophic factors may be required by different types of neurons, from the peripheral and central nervous systems, for survival and functional maintenance *in vivo* and *in vitro* (for reviews see Varon and Adler, 1981; Varon et al., 1983b, 1984; Manthorpe and Varon, 1985). Nerve growth factor (NGF), the best characterized neuronotrophic factor, supports the survival of embryonic sensory and sympathetic neurons (e.g., Greene and Shooter, 1980). The composition of 7S NGF from mouse submaxillary glands and the amino acid sequence of its biologically active subunit, β -NGF, are known (for review see Bradshaw and Costini, 1983). A second neuronotrophic factor, distinct from NGF, was isolated from pig brain

(Barde et al., 1982). It has been reported to have an M_r of about 12,300, and to support the survival of embryonic day (E) 10 to E15 chick dorsal root but not of sympathetic or ciliary ganglionic neurons.

A ciliary neuronotrophic factor (CNTF) has recently been purified from selected chick eye tissues on the basis of its being required for the *in vitro* survival of neurons from E8 chick ciliary ganglia (Barbin et al., 1984; Manthorpe and Varon, 1984). The purified chick CNTF also displays trophic activity for avian and rodent sensory and sympathetic ganglionic neurons. It has an apparent M_r of 20,400 and is distinct from NGF and the pig brain factor with regard to both its target neurons and its molecular properties. CNTF activity is present in high amounts in adult rat sciatic nerve extracts (Williams et al., 1984) and also has been found to accumulate locally in neonatal and adult rat brain in response to experimentally induced lesions (Manthorpe et al., 1983b; Nieto-Sampedro et al., 1983).

Trophic titers of various factors are determined using *in vitro* bioassays (for review see Varon et al., 1983a). The neuronal target cells are incubated with serial dilutions of the trophic factor source, and the number of neurons surviving at selected culture times reflects the concentration of the trophic factor in the test culture. We describe here a new method to recognize polypeptides in tissue extracts that have trophic activity for ciliary ganglionic neurons. The polypeptides of the crude material are separated by sodium dodecyl sulfate (SDS)-PAGE and electrophoretically transferred to nitrocellulose. The resulting blots are then incubated with purified neurons from E8 chick ciliary ganglia to probe for CNTF activities. After 24 hr of incubation, living neurons are identified using a vital stain. Only those neurons supplied with CNTF will survive on these nitrocellulose blots and, thus, the CNTF-supported, dye-stained neurons define a band coincidental with the electrophoretic position of the CNTF polypeptide. This demonstrates that CNTFs retain their trophic activity throughout electrophoresis and subsequent binding to nitrocellulose. Using this technique, the electrophoretic behaviors of CNTFs from chick eye and rat nerve extracts and from rat brain lesion fluid are compared.

Materials and Methods

SDS-PAGE and transfers of proteins to nitrocellulose. Polypeptides were separated by SDS-PAGE according to the method of Laemmli and Favre (1970) using a 7.5 to 20% polyacrylamide gradient, and then were transferred electrophoretically to nitrocellulose paper (Millipore, 0.45- μ m pore size) according to the method of Towbin et al. (1979). Transfer was performed at 500 mA for 2 hr, 150 mA for 12 to 18 hr, and finally 600 mA for 1 hr without cooling. The distance between electrodes was 3 cm.

Incubation of blots with purified ciliary ganglionic neurons. After electrophoretic transfer of the proteins, the nitrocellulose sheet was rinsed with phosphate-buffered saline (PBS) and cut into strips (0.5 $\times$ 10 cm) containing individual electrophoretic lanes. Unoccupied protein-binding sites were blocked with 1% ovalbumin-PBS, pH 7.2, for 2 hr at room temperature in 15-ml capped plastic tubes. Each blot was placed in a shallow rectangular trough (14 $\times$ 1 $\times$ 1 cm) cut into a block of plastic. The nitrocellulose strips were immobilized flat on the bottom of their trough, and blotted surface facing up, with rings of plastic tubing (outer diameter, 1 cm) that were

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wedged against the sides and bottom of the trough at each end of the nitrocellulose strip. The troughs and tubing were sterilized before use by brief treatment with 70% ethanol and UV irradiation. Once in place, the blots were washed for 15 to 20 min with culture medium (Dulbecco's modified Eagle's medium supplemented to 26.4 mM NaHCO₃, 2 mM glutamine, 10% fetal calf serum, and 100 units/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of Fungizone).

Ciliary ganglia from E8 chick embryos were dissociated into a single cell suspension in culture medium as described (Varon et al., 1979), and its neurons were enriched (to better than 90% purity) by differential cell attachment for 2 hr at 37°C on tissue culture plastic (Davis et al., 1985). Aliquots of the neuronal cell suspension (2×10^5 neurons in 3 ml of culture medium) were evenly distributed with a Pasteur pipette over each nitrocellulose strip. The troughs were covered with a sterile plastic lid, placed on a level tray, and incubated at 37°C in humidified 95% air/5% CO₂ for 3 or 24 hr. In some experiments wider blots containing multiple electrophoretic lanes were placed in larger troughs (14 × 4 × 0.8 cm) and seeded with 5×10^5 neurons in 15 ml of medium. The surviving neurons were visualized by their ability to form a blue formazan reaction product from a yellow substrate, 13-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co., M2128), as described by Mosmann (1983). After 22 hr of culture, a solution of MTT-PBS (5 mg/ml) was added to the medium in a volume ratio of 1:10 and gently mixed. The neurons were incubated for another 2 to 3 hr and then fixed on the nitrocellulose strips by first adding an equal volume of 2% glutaraldehyde in Eagle's basal medium. After 15 min, the fixative was replaced with fresh glutaraldehyde medium and the fixation was continued for an additional 30 min. The fixed cultures on nitrocellulose could be stored in PBS at 4°C for at least a week. The number of blue-labeled neurons was determined visually within 1-mm² areas by direct counts using light microscopy.

Preparation of tissue extracts. E15 chick intraocular tissues including the choroid, iris-ciliary body, and pigment epithelium (CIPE) were dissected and extracted with water as described previously (Manthorpe et al., 1980). Chick CNTF was purified from CIPE extract according to the method of Barbin et al. (1984) and had a specific activity of 7.7×10^6 trophic units (TU)/mg of protein. Adult rat sciatic nerve extracts, prepared as described by Williams et al. (1984), were concentrated 10-fold using Amicon PM10 ultrafiltration. Gelfoam sponges placed in adult rat entorhinal lesions were collected at 16 days post-lesion and extracted as described by Nieto-Sampedro et al. (1983).

Results

Survival of ciliary ganglionic neurons on nitrocellulose paper. The *in vitro* behavior of E8 chick ciliary ganglionic neurons seeded on ovalbumin-blocked, unblotted nitrocellulose strips is illustrated in Figure 1. Neuronal attachment was allowed to proceed for 3 hr, and then the MTT was added. After dye exposure, the neurons appeared

as round blue cells which were evenly distributed on the nitrocellulose, regardless of the presence of purified chick eye CNTF in the culture medium (Fig. 1A). Neuronal cell density and morphologies appeared unchanged after 24 hr in cultures containing CNTF at 100 TU/ml (Fig. 1B). In sharp contrast, very few neurons were stained with MTT after 24 hr in cultures from which CNTF had been omitted (Fig. 1C). Two representative fields of CNTF-supported and CNTF-deprived cultures are shown at higher magnification in Figure 1, D and E, respectively. Note the round cell appearance of the dye-positive, viable neurons. The same neurons, if cultured on laminin-treated polyornithine substrate (Manthorpe et al., 1983a), would display neuritic outgrowth and, in fact, will even do so in CNTF-supported cultures on nitrocellulose which has been pretreated with laminin (data not shown). The ability of these cells to form neurites, as well as the preponderance (>90%) of neurons in the seeded cell population, allowed us to conclude that (i) E8 ciliary ganglionic neurons may be cultured on nitrocellulose substrata and (ii) their survival in this culture system, as in all previously examined culture systems, continues to depend on the availability of CNTF.

Trophic competence of CNTF supplied in a substratum-bound form. We wished to determine whether nitrocellulose-bound chick eye CNTF would retain its ability to provide trophic support to target neurons. Nitrocellulose strips were soaked for 1 hr in 3 ml of culture medium containing purified CNTF (100 TU/ml) and were rinsed with three changes of PBS for 2 to 3 hr; the remaining protein sites were blocked with ovalbumin. The strips were then placed in their troughs and supplied with test neurons in CNTF-free medium. After a 24-hr incubation, the cultures were treated with the dye and examined. Figure 2A demonstrates the substantial presence of dye-positive, viable neurons in these cultures. Neuronal morphologies in these cultures appear similar to those in cultures containing CNTF in the media (cf. Fig. 1B).

To examine whether CNTF might be acting locally after binding to the substratum, a small volume (2 µl) of CNTF at 10⁶ TU/ml was "spotted" onto nitrocellulose membranes, the membranes were blocked with ovalbumin, and ciliary neurons were seeded over the entire strip and cultured for 24 hr in CNTF-free medium. Figure 2, B and C, shows, respectively, a CNTF-spotted nitrocellulose area after staining with amido black (but without cell seeding) and tested with the vital dye after neuronal culture. Only the CNTF-spotted area contained dye-positive neurons (higher magnification photographs of a spotted and an unspotted area are shown in Fig. 2, D and E),

Figure 1. Recognition of dissociated neurons cultured on nitrocellulose paper in the presence or absence of CNTF in the culture medium. Purified ciliary ganglionic neurons were cultured on nitrocellulose paper, stained using a vital dye, fixed with glutaraldehyde, and photographed using light microscopy as described under "Materials and Methods." Typical cultures are shown 3 hr after seeding with no CNTF added (A), 24 hr with 100 TU/ml of purified chick eye CNTF in the medium (B), and 24 hr with no CNTF added (C). Higher magnifications of the cultures in B and C are shown in D and E, respectively. Note the obvious decrease in the number of stained viable neurons in C and E. Magnification bars represent 1.0 mm in A to C and 100 µm in D and E.

Figure 2. Survival of dissociated neurons on nitrocellulose paper containing bound purified chick eye CNTF. Purified ciliary neurons were cultured for 24 hr in the absence of CNTF in the medium on strips of nitrocellulose which had been previously treated with purified chick eye CNTF. *A*, Ciliary cultures (24 hr) on strips of nitrocellulose which had been uniformly treated with CNTF. *B*, Amido black stain of a piece of nitrocellulose "spotted" with 2 μ l of purified CNTF (130 μ g/ml of protein and 10^6 TU/ml). *C*, Ciliary culture (24 hr) on a nitrocellulose strip (also spotted with 2 μ l of purified CNTF). Note the localized neuronal survival only on the area containing bound CNTF. Higher magnifications of the culture in *C* are shown in *D*, an area within the spot, and in *E*, an area outside the spot. Ciliary neurons cultured on CNTF-spotted paper for 3 hr in either the presence or absence of CNTF in the medium, or for 24 hr in the presence of CNTF, appeared identical to the culture shown in *A*. Magnification bars represent 1.0 mm in *A* to *C* and 100 μ m in *D* and *E*.

indicating that survival of ciliary ganglionic neurons was restricted to the area where CNTF was previously spotted. The dye-positive neurons appeared similarly distributed on both spotted and unspotted areas (i.e., the cultures appeared the same as shown in Figs. 1*B* and 2*A*) at either 3 hr with or without CNTF in the medium or at 24 hr with CNTF included in the medium (data not shown).

The number of dye-positive neurons per unit area was determined using light microscopy, and the results are shown in Figure 3. Neuronal density values are given for (i) a 3-hr culture on CNTF-free nitrocellulose and in a CNTF-free medium, illustrating the neuronal cell density shortly after attachment; (ii) a 24-hr culture on CNTF-free nitrocellulose in the presence of CNTF-supplemented medium (showing the distribution of viable neurons over the entire strip); and (iii) and (iv) a 24-hr culture in CNTF-free medium but with a CNTF-spotted nitrocellulose, where neuronal counts were taken within or outside the CNTF-spotted area, respectively. Such measurements clearly indicate that the neuronal cell attachment was independent of either free or bound CNTF. CNTF-dependent neuronal survival was equally promoted by supplying soluble CNTF in the medium or bound CNTF on the nitrocellulose. In the latter case, neuronal survival was precisely restricted to the bound-CNTF area.

CNTF within a crude extract can be recognized after SDS-PAGE and transfer to nitrocellulose. The principle of localized neuronal survival on immobilized CNTF was applied to the detection of CNTF in tissue extracts. Different amounts of chick ocular (CIPE) extract were submitted to SDS-PAGE and blotted to nitrocellulose. The blots were then probed with ciliary ganglionic neurons in CNTF-free medium. The results, shown in Figure 4, indicate that there was a well defined band, migrating at about 20,000 daltons, which supported the survival of ciliary ganglionic neurons. This apparent molecular weight corresponds to the reported $M_r = 20,400$ of purified chick CNTF (Barbin et al., 1984). Indeed, purified chick CNTF subjected to SDS-PAGE and blotting occupies a band (revealed by a localized area of surviving neurons) which co-migrates with the 20,000 dalton band from CIPE extracts (data not shown). Neither the purified eye CNTF nor the CIPE extract revealed other CNTF bands with trophic activity for ciliary neurons (cf. Fig. 4*B*). The intensity and width of the blot band defined by dye-positive cells (Fig. 4*A* and inset to Fig. 4*B*) decreased when less extract was loaded on the gels to be blotted. It is possible to scan the blots

along their length using light microscopy, readily identify the regions where "blue" cell density exceeds background, and count dye-positive neurons across the nitrocellulose strips over the positive band region. The greatest neuronal density within the dye-positive area defines a peak representing the electrophoretic mobility of the chick eye CNTF polypeptide. The peak amplitude and area decrease as one decreases CIPE extract loads on the original SDS gel. The study indicates that loads of 10 TU (<2 ng of protein) of CNTF per lane, whether in the crude CIPE extract or in the purified form, can be detected by this method.

Comparison of CNTFs in different crude sources. Figure 5 compares cultures on blots obtained after SDS-PAGE of two other CNTF sources besides the chick eye. Extracts of adult rat sciatic nerve are particularly rich in CNTF activity (Williams et al., 1984). Mechanical lesions in the cerebral cortex of young or adult rats lead to an accumulation of CNTF activity in both the wound area and the injured brain tissue (Manthorpe et al., 1983b; Nieto-Sampedro et al., 1983). The molecular nature of either of these rat CNTFs has not yet been defined. Use of the present SDS-PAGE and blot approach has revealed, from both rat sources, the occurrence of a 24,000 dalton CNTF protein distinct in electrophoretic mobility from the chick eye CNTF. In one experiment, the rat nerve extract also displayed a second active CNTF band in the 19,000 dalton range distinct from both the 24,000 and 20,000 dalton bands described above. Note, however, that the amount of sciatic nerve extract loaded in this experiment (containing 7000 TU) was relatively high. Indeed, in two other runs using independently prepared extracts, lower activity loads (300 TU) demonstrated only the 24,000 dalton CNTF band (data not shown). The relationship of the CNTF polypeptides from rat sciatic nerve extracts and rat brain lesion fluids to chick eye CNTF remains to be determined. Nevertheless, these findings illustrate that the present blot and culture method can be used to recognize distinct CNTF species within the same or different sample materials.

Discussion

In the present report we have used SDS-PAGE and nitrocellulose blotting to identify and characterize proteins which support ciliary ganglionic neuronal survival. Our methodological approach was based on that used by Hayman et al. (1982), who established the

Figure 3. Quantitation of neuronal survival on nitrocellulose paper containing bound purified chick eye CNTF. Ciliary neurons, cultured for 24 hr on CNTF-spotted papers similar to those shown in Figure 2, were counted within sampling areas using light microscopy, and the number of neurons per square millimeter area was determined. Neuronal densities are shown for (1) 3-hr cultures without added CNTF, (2) 24-hr cultures on CNTF-spotted nitrocellulose with CNTF in the culture medium, (3) 24-hr cultures on CNTF-spotted nitrocellulose but without CNTF in the medium, and (4) 24-hr cultures on areas away from the CNTF spot (without CNTF in the culture medium). Values are given $\pm$ SD (n = the number of individual areas counted).

principle that SDS-PAGE blots of cell attachment-promoting proteins could be probed with normal rat kidney cells to identify biologically active individual polypeptides from plasma.

Methodological prerequisites. The present study used E8 chick ciliary ganglionic neurons as probe cells to detect chick and rat CNTFs on nitrocellulose. Three prerequisites were satisfied which permitted the blot system to be successful. *First* was the demonstration that these neurons attach to nitrocellulose substrata, and that they will survive there only when supplied with a trophic factor in the culture medium. It is reasonable to expect that other probe neurons will behave similarly if provided with an appropriate neurotrophic agent. *Second* was the ability of the trophic factor to retain its activity after exposure to SDS and β -mercaptoethanol during the electrophoretic steps. Purified chick eye CNTF had been shown previously to survive such treatments (Barbin et al., 1984), but there is no assurance that all trophic factors will share this stability. It will be interesting in this regard to adapt the current approach to blots

obtained from SDS-free gels, e.g., native PAGE or isoelectric focusing PAGE. The *third* prerequisite for success in the present method was the capability of a trophic factor to express its activity in culture after having been bound to nitrocellulose.

Applications of the present methodology. The present blot and culture technique, particularly if applied to non-denaturing gel electrophoretic systems, may offer unique experimental approaches to several important questions concerning ciliary ganglionic neurotrophic factors. A large number of different source materials display CNTF activity (cf. Manthorpe and Varon, 1984) and, as illustrated in Figure 5, the present technique can be used to compare the electrophoretic behaviors of different CNTFs. The technique is particularly well suited for examining CNTFs in samples containing widely different protein and activity contents. This technique should be useful for the detection of apparent molecular weight differences among CNTFs taken from the same tissue at different developmental ages or after experimental treatments of the source cells. Con-

Figure 4. Recognition of nitrocellulose-bound CNTF within an aqueous extract of chick embryo eye. Adjacent individual lanes containing different amounts of CIPE extract were transferred to nitrocellulose after SDS-PAGE, and the resulting blots were probed for CNTF activity using purified ciliary ganglionic neurons as described under "Materials and Methods." *A*, Photograph of the fixed blot after culture showing ciliary neuronal survival localized only to one discrete band. Each individual lane was loaded with CIPE extract (400 μg/ml of total protein and 8000 TU/ml of CNTF activity) containing different amounts of CNTF: lane 1, 7 TU (1 ng); lane 2, 15 TU (2 ng); lane 3, 30 TU (4 ng); lane 4, 60 TU (8 ng); lane 5, 120 TU (16 ng); lane 6, 240 TU (32 ng); lane 7, amido black stain of blotted CIPE extract (240 TU and 12 μg of total protein per lane). *B*, Neuronal number per 1 × 5 mm strip width across each blot lane is shown with respect to the distance from the gel origin. The inset is a higher magnification photograph of the corresponding neuron-containing lanes shown in *A*. Molecular weight standards, run in adjacent lanes, were used to plot the standard curve.

versely, the procedure will allow the detection of apparent molecular weight changes (not accompanied by inactivation) after different treatments are applied to a purified CNTF before electrophoresis and blot analysis. Also, blots containing a CNTF could be exposed to experimental treatments after electrophoresis and blotting but before neuronal seeding to gain information on the stability of the bound CNTF activity.

The present technique might be adapted to the use of different neurons and to different neuronotrophic factors, thus extending the applicability to each of the above analyses. Trophic activities for different neurons have been observed in the same source material, and the modified approach would permit one to ascertain whether the activities rest with the same or with different polypeptides. Alternatively, the methodology might be adapted to the investigation of neurite-promoting or other neuron-modulating proteins, as well as to proteins affecting culture behaviors of glial or other probe cells.

Soluble versus bound CNTF. The present findings draw attention

to an important question, namely, whether CNTF blotted to nitrocellulose expresses its activity *while remaining bound* or *only upon release* from its initially bound state. The rather strict localization of living neurons to the blotted CNTF excludes the possibility of a general release of the factor into the medium but does not entirely rule out the possibility of a limited local release of trophic molecules. This latter possibility, however, seems unlikely based on two observations (data not shown), namely, that (i) CNTF blots underwent no apparent loss of trophic competence even after having been stored for 3 weeks in PBS before the presentation of probe neurons, and (ii) treatments of CNTF blots with either 90% methanol or 8 M urea overnight also failed to impose detectable reductions in trophic activity on the blot. This evidence favors (but does not prove) the view that, in this culture system, the probe neurons *encounter* the CNTF molecules in their bound state, rather than in the local humoral environment. The possibility of an immobilized trophic factor supporting neuronal survival in culture, also explored with NGF (e.g.,

Figure 5. Recognition of nitrocellulose-bound CNTF within aqueous extracts of chick embryo eye, adult rat sciatic nerve, and Gelfoam sponges from adult rat brain lesions. Experimental conditions were as in Figure 4 except that ciliary neurons were cultured on nitrocellulose blots from SDS gels containing three adjacent sample lanes. A, Photograph of the fixed blot after culture showing neuronal survival on discrete areas: lane 1, nerve extract (40 μ g of protein and 7000 TU/lane); lane 2, CIPE extract (20 μ g and 400 TU); and lane 3, brain lesion fluid (200 μ g and 100 TU). B, Neuronal numbers per 1 $\times$ 5 mm strip across each blot lane are shown with respect to the distance from the gel origin. Inset in B, Larger magnification of the area in A containing localized ciliary neuronal survival. Molecular weight standards, run in adjacent lanes, were used to plot the standard curve. C, Amido black stain of each extract containing the same amounts of protein indicated in A.

Frazier et al., 1973; Oger et al., 1974), raises this interesting question of whether such a capability also exists *in vivo*.

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