# UC Irvine
## UC Irvine Previously Published Works

**Title**

Response of purified chick motoneurons to myotube conditioned medium: Laminin is essential for the substratum-binding, neurite outgrowth-promoting activity

**Permalink**

https://escholarship.org/uc/item/22p2c02v

**Journal**

Neuroscience Letters, 59(2)

**ISSN**

0304-3940

**Authors**

Calof, Anne L
Reichardt, Louis F

**Publication Date**

1985-08-01

**DOI**

10.1016/0304-3940(85)90197-1

Peer reviewed

# RESPONSE OF PURIFIED CHICK MOTONEURONS TO MYOTUBE CONDITIONED MEDIUM: LAMININ IS ESSENTIAL FOR THE SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY

ANNE L. CALOF* and LOUIS F. REICHARDT

*Division of Neuroscience, Department of Physiology, University of California School of Medicine, San Francisco, CA 94143 (U.S.A.)*

Purified motoneurons from chick, cultured on polycationic substrata treated with myotube-conditioned (MCM), respond by rapidly extending neurites. When MCM, partially purified by salt precipitation and ion-exchange chromatography, was fractionated on Sepharose CL-4B, the peak of neurite outgrowth-promoting activity (NOPA) corresponded to a peak of laminin (LA) immunoreactivity. Fractions from this peak contained a protein band that comigrated with an LA standard on sodium dodecyl sulfate gels. Antibodies to LA immunoprecipitated all motoneuron NOPA from MCM, and the specifically immunoprecipitated material comigrated with LA in both reducing and non-reducing gels. It thus appears that LA in MCM is essential for the ability of this conditioned medium to promote motoneuron neurite outgrowth.

The importance of muscle for the embryonic development of motoneurons, in processes such as naturally occurring motoneuron death and motor nerve extension, has been well demonstrated in vivo [12, 17]. Knowledge of this work has led many investigators to examine the effects of muscle extracts or medium conditioned over cultures of myotubes on the growth of cultured whole spinal cord cells or explants, in an attempt to discover molecules important for motoneuron development [4, 5, 13, 15]. However, the interpretability of such studies suffers from the fact that motoneurons comprise a very small percentage of cells in the spinal cord. Even when motoneurons are identified in cultures from whole spinal cord [1], it is not possible to distinguish direct effects of exogenous substances on motoneurons from effects mediated indirectly by other classes of spinal cord cells.

The development of techniques for purifying embryonic chick spinal motoneurons has recently made cultures of purified motoneurons available for study [3, 16]. We have reported that purified motoneurons respond to two distinct activities in myo-

*Author for correspondence at present address: Howard Hughes Medical Institute, Center for Neurobiology and Behavior, Columbia University College of Physicians and Surgeons, New York, NY 10032, U.S.A.

tube-conditioned medium (MCM): a neurite outgrowth-promoting activity (NOPA), which binds to the culture substratum and acts to promote rapid, profuse outgrowth of motoneuron processes; and a survival-enhancing activity, which acts over the course of a week in culture to promote motoneuron survival [3]. Further characterization of the substratum-binding NOPA by biochemical and immunochemical techniques, reported below, has demonstrated that laminin (LA) is essential for the ability of MCM to promote motoneuron neurite outgrowth.

Motoneurons were purified from chick embryos by retrograde axonal transport of a fluorescent dye and fluorescence-activated cell sorting [3]. Tissue culture plates (96-well; Costar) were exposed to poly-D-lysine and treated with samples to be tested for NOPA (50 $\mu$l/well) overnight at 4°C. Wells were washed and motoneurons plated into them at a density of 3000–5000 cells/well and maintained for 24 h in serum-free growth medium [3].

MCM was obtained from chick myotube cultures established according to the method of Konigsberg [7] and maintained as described [3]. After fusion of myotubes, cultures were periodically treated with 10 $\mu$M cytosine-$\beta$-D-arabinofuranoside to suppress fibroblast growth. MCM was prepared by washing cultures twice in plain Dulbecco's Modified Eagle's Medium containing 0.45% glucose (DME), then by incubation in DME supplemented with 1% horse serum (HS), 25 $\mu$g/ml ovotransferrin (Otf) [3], 2 mM glutamine, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. Seven to 8 ml of medium per 100-mm diameter tissue culture plate were conditioned for 3–5 days and harvested. Metabolically labelled MCM was prepared by rinsing cultures with methionine (MET)-free DME, then labelling in MET-free DME supplemented with 1% HS, [35S]MET (40 $\mu$Ci/ml, 1110 Ci/mmol; Amersham), unlabelled MET (final concn. 21 $\mu$M), 25 $\mu$g/ml Otf and glutamine and antibiotics as above. Cultures were maintained for 2 days, then fed with 1 ml of MET-containing DME. After 24

→

Fig. 1. [35S]MET-labelled MCM was concentrated and fractionated on a column of Sepharose CL-4B as described in the text. The column was calibrated with LA and $\beta$-galactosidase. High-molecular-weight aggregates of $\beta$-galactosidase served to mark the void volume ($V_0$), and 35SO$_4$ was used to mark the total included volume ($V_t$). The top panel of this figure shows enzyme-linked immunosorbent assays (ELISA), of LA-IR in column fractions. A rabbit antiserum to purified mouse LA [20] was generated, and affinity-purified antibodies were prepared by chromatographing the serum on LA coupled to Affigel 10 (Bio-Rad) at 0.8 mg/ml gel. ELISAs [6] were performed on column fractions applied to polylysine-coated tissue culture wells. using affinity-purified anti-LA antibodies at 1.5 $\mu$g/ml. Results were quantified spectrophotometrically at 415 nm. Error bars show the mean and range of two determinations. The second panel shows motoneuron neurite outgrowth, assayed by growing purified motoneurons on polylysine-coated substrata that had been treated with column fractions as described in the text. Neurite outgrowth was quantified as the percentage of cells bearing neurites $\geq$ 2 cell diameters in length. Over 200 cells were counted per culture; data are presented as the mean and range of two cultures. The SDS–polyacrylamide gel (PAG) system of Laemmli [8] was used for the gel shown in the bottom panel. $\beta$-Mercaptoethanol was omitted. The separating gel was a 3% to 15% exponential acrylamide gradient, and the stacking gel was 2.8% acrylamide. The gel was cast on a GelBond PAG solid support (Marine Colloids). Labelled material was detected by fluorography using preflashed Kodak XAR-2 film at −70°C [2]. Standards for this non-reducing gel, shown in the two outside lanes, were [125I]LA and 125I-labelled mouse immunoglobulin G (IgG).
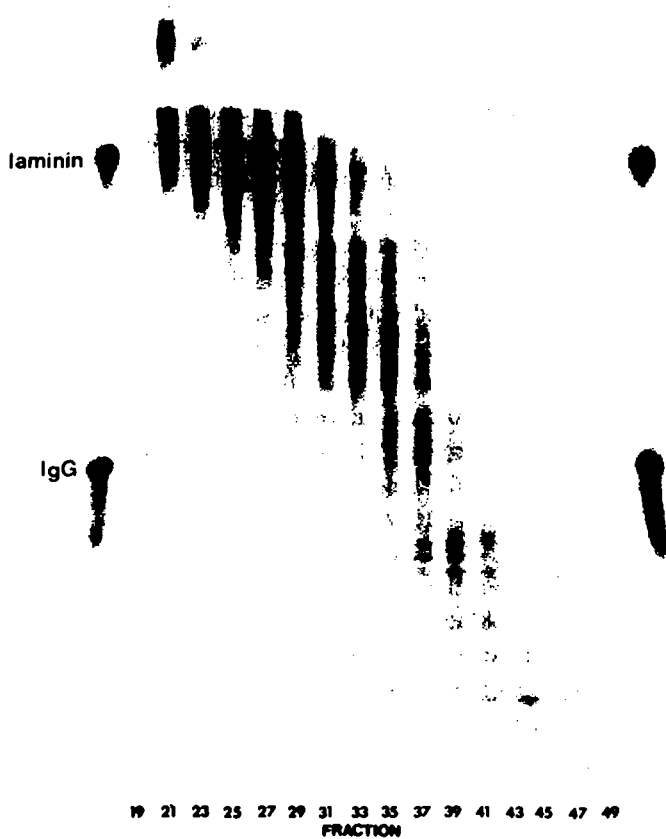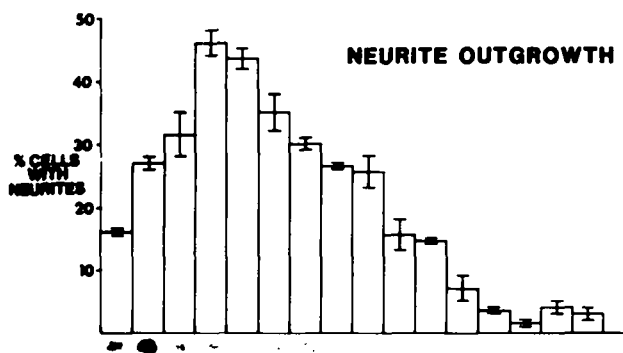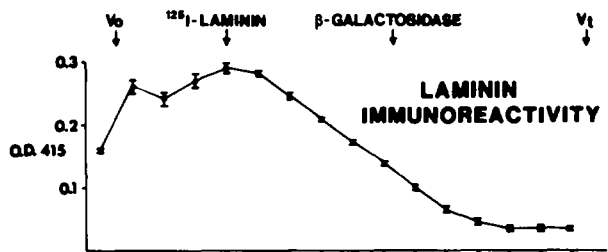
h, the labelled MCM was harvested, filtered (0.2 μm) and treated with the protease inhibitors: phenyl-methylsulfonyl fluoride (PMSF; 1 mM), ethylenediaminetetraacetic acid (EDTA, 3 mM), pepstatin ($10^{-7}$ M) and N-ethyl maleiimide (NEM; 2 mM).

The motoneuron NOPA from MCM was fractionated as follows: 90 ml of MCM, metabolically labelled with [$^{35}$S]MET, were mixed with 130 ml of 'cold' MCM, and the pooled conditioned medium was depleted of fibronectin on gelatin–Sepharose [19]. Without this step, insoluble aggregates formed during subsequent procedures and NOPA was lost. The MCM was brought to 45% saturating ammonium sulfate, and precipitated material was removed by centrifugation and dialysed against 0.1 M NaCl–0.05 M Tris-HCl, pH 7.4. The dialysed material (7 ml) was mixed overnight with 2 ml of diethylaminoethyl (DEAE) cellulose (Whatman DE52). Neither the supernatant from the ammonium sulfate precipitation nor the unbound material from the ion-exchange step had NOPA. Material eluted from the DEAE–cellulose with 1 M NaCl–0.05 M Tris-HCl, pH 7.4., contained the NOPA, and this fraction (1.3 ml) was chromatographed on a column (28 × 1.5 cm) of Sepharose CL-4B equilibrated in 0.5 M NaCl–0.05 M Tris-HCl (pH 7.4) containing 0.2 mg/ml hemoglobin–0.1 % Triton X-100–2 mM EDTA–1 mM PMSF–$10^{-7}$ M pepstatin–2 mM NEM. Alternate 1-ml fractions were tested for substratum-binding NOPA and LA immunoreactivity (IR) and run on SDS gels (Fig. 1). The peak of activity corresponded both to the presence of a protein which comigrated with authentic LA in the gel, and to a peak of LA-IR. Photographs of motoneurons grown for 24 h on substrata treated with different column fractions demonstrate the extensive neurite outgrowth seen with positive fractions (Fig. 2B). Thus, LA appears to be present in the fractions of MCM that have the ability to promote neurite outgrowth by purified motoneurons.

The availability of anti-LA antibodies suggests further experiments to confirm the association of LA with the motoneuron NOPA in MCM, such as attempt to block motoneuron neurite outgrowth on MCM-treated culture substrate by introducing antibodies into the culture medium. However, because the antibodies available were generated using mouse LA, it seemed possible that they would not block the effects of the chick LA in MCM; indeed, we have performed such experiments and they have not succeeded [3]. Nevertheless, because the profile of LA-IR in Fig. 1 indicates that at least some anti-mouse LA antibodies recognize chick LA, anti-mouse LA antibodies should at least be able to *remove* LA from MCM. Accordingly, MCM was subjected to immunoprecipitation with affinity-purified anti-LA antibodies, in order to determine whether removal of LA results in the disappearance of the motoneuron NOPA. MCM (0.4 ml) was mixed with 10.8 μg affinity-purified anti-LA-antibodies and carrier non-immune Ig (24 μg). The mixture was incubated overnight at 4°C, and goat anti-rabbit Ig (Cappel) was added to the equivalence point (determined to be 25 μg/μl carrier Ig) for a further 24 h at 4°C. The precipitate was removed by centrifugation.

Motoneuron NOPA was indeed removed from MCM by this procedure (Fig. 3). In order to determine whether the immunoreactive component removed from MCM was, in fact, LA, identical immunoprecipitations were performed using [$^{35}$S]MET-

labelled MCM. The precipitated material (washed once in 0.1 M NaCl–0.05 M Tris-HCl, pH 7.4) was subjected to electrophoresis under reducing and non-reducing conditions. In non-reducing gels, the specifically immunoprecipitated material appears
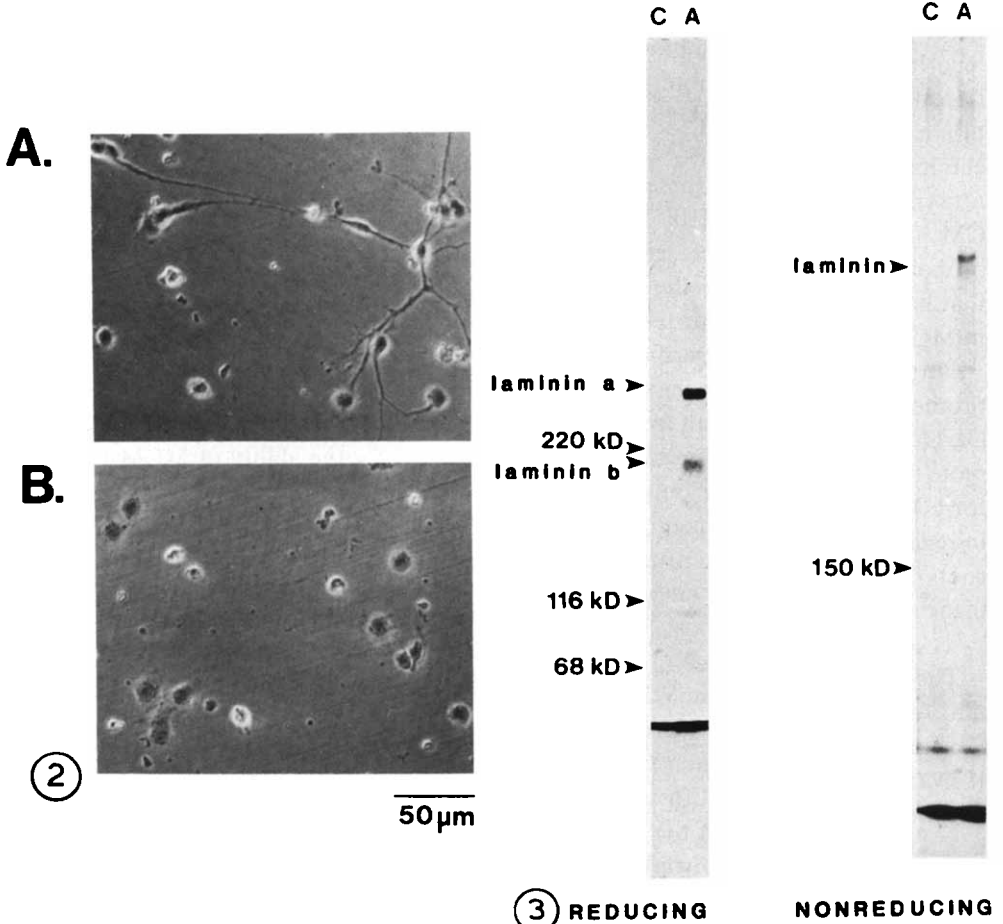


Fig. 2. (left) Motoneurons were grown on polylysine substrata treated with fractions from the column illustrated in Fig. 1 (see text for culture conditions). After 24 h in culture, the cells were fixed and washed as previously described [3]. A: motoneurons grown on substratum treated with fraction 27. B: motoneurons grown on substratum treated with fraction 47.

Fig. 3. (right) MCM was subjected to immunoprecipitation with or without affinity-purified anti-LA antibodies (prepared as described in the legend to Fig. 1). The results of motoneuron neurite outgrowth assays of substrata treated with the supernatants of these immunoprecipitations, expressed as the percent of cells with neurites, were: control (without anti-LA), 27 ± 5; anti-LA, 5 ± 1. The proteins removed from MCM by immunoprecipitation were identified by SDS-PAG-analysis of identical immunoprecipitates from [35S]MET-labelled MCM. Separating gels were 3% to 15% exponential acrylamide gradients, with 2.8% stacking gels, as described in the legend to Fig. 1. Adjacent lanes from reducing (with β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) gels are shown. C, control immunoprecipitation (anti-LA antibodies omitted); A, experimental immunoprecipitation (affinity-purified anti-LA antibodies were used). The molecular weight standards for the non-reducing gel were mouse laminin (850–1000K; K = kdalton) and mouse IgG (150K). Standards for the reducing gel were mouse LA heavy chain ('laminin a', 400K), cellular fibronectin (220K), LA light chain ('laminin b', 200–220K), β-galactosidase (116K), and bovine serum albumin (68K). Percent of cells with neurites: control, 27 ± 5; anti-LA, 5 ± 1.

as a high-molecular-weight protein band whose position is consistent with its identification as LA. This band is converted, upon reduction, to two bands with $M_r$s of $\sim 400,000$ and $\sim 200,000$, appropriate for the heavy and light chains of LA.

An earlier characterization of the motoneuron NOPA from MCM indicated that it was similar in its mode of action, size, density and enzymatic susceptibilities to a 'neurite outgrowth-promoting factor' from bovine corneal endothelial cell conditioned medium (BCE-CM) [3, 9]. It has recently been reported that immunoprecipitation of LA from BCE-CM, as well as several other types of conditioned media, eliminates the ability of these conditioned media to promote neurite outgrowth by rat sympathetic neurons [10]. In addition, purification of the active factor from BCE-CM has confirmed the presence of LA [11]. Those authors suggested that LA was probably responsible for the substratum-binding NOPA of all conditioned media that have this activity. Our results indicate that this is indeed the case for the NOPA in MCM that acts upon purified chick spinal motoneurons.

Purified LA, derived from extracellular matrix [20], has previously been shown to promote neurite outgrowth from many types of neurons [14], including motoneurons [3]. Our finding that the presence of LA is essential for the ability of MCM to promote motoneuron neurite outgrowth is consistent with this result and suggests a role for LA in directing motoneuron process outgrowth in vivo. The identification of LA in regions of motoneuron process outgrowth in vivo (such as the spinal cord ventral roots) during periods in embryonic development in which this outgrowth is commencing [18] further supports this idea.

1 Bennett, M.R., Lai, K. and Nurcombe, V., Identification of embryonic motoneurons in vitro: their survival is dependent on skeletal muscle, Brain Res., 190 (1980) 537–542.

2 Bonner, W.M. and Laskey, R.A., A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels, Eur. J. Biochem., 46 (1974) 83–88.

3 Calof, A.L. and Reichardt, L.F., Motoneurons purified by cell sorting respond to two distinct activities in myotube conditioned medium, Dev. Biol., 106 (1984) 194–210.

4 Dribin, L.B. and Barrett, J.N., Two components of conditioned medium increase neuritic outgrowth from rat spinal cord explants, J. Neurosci. Res., 8 (1982) 271–280.

5 Henderson, C.E., Huchet, M. and Changeux, J.-P., Neurite-promoting activities for embryonic spinal neurons and their developmental changes in the chick, Dev. Biol., 104 (1984) 336–347.

6 Hudson, L. and Hay, F.C., Practical Immunology, Blackwell, Oxford, 1980, 359 pp.

7 Konigsberg, I.R., Skeletal myoblasts in culture, Meth. Enzymol., 58 (1979) 511–526.

8 Laemmli, U.K., Cleavage of structural protein during the assembly of the head of bacteriophage T4, Nature (Lond.), 227 (1970) 680–685.

9 Lander, A.D., Fujii, D.K., Gospodarowicz, D. and Reichardt, L.F., Characterization of a factor that

promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan, J. Cell. Biol., 94 (1982) 574–585.

10 Lander, A.D., Fujii, D.K. and Reichardt, L.F., Laminin is associated with the 'neurite outgrowth-promoting factors' found in conditioned media, Proc. Natl. Acad. Sci. USA, 82 (1985) 1882–1890.

11 Lander, A.D., Fujii, D.K. and Reichardt, L.F., Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules, J. Cell. Biol., 101 (1985) in press.

12 Lewis, J., Chevallier, A., Kieny, M. and Wolpert, L., Muscle nerves do not develop in chick wings devoid of muscle, J. Embryol. Exp. Morphol., 64 (1981) 194–210.

13 Longo, F.M., Manthorpe, M. and Varon, S., Spinal cord neuronotrophic factors (SCNTFs). I. Bioassay of schwannoma and other conditioned media, Dev. Brain Res., 3 (1982) 277–294.

14 Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F.M., Davis, G.E. and Varon, S., Laminin promotes neuritic regeneration from cultured peripheral and central neurons, J. Cell Biol., 97 (1983) 1882–1890.

15 Nurcombe, B., Hill, M.A. and Bennet, M.R., Motor neuron survival and neuritic extension from spinal cord explants induced by factors released from denervated muscle, Brain Res., 291 (1984) 19–28.

16 Okun, L.M., Identification and isolation in vitro of neurons marked in situ by retrograde transport. In D. Gottlieb (Ed.), New Approaches in Developmental Neurobiology, Society for Neuroscience, Bethesda, MD, 1981, pp. 109–121.

17 Oppenheim, R.W., Neuronal death and some related regressive phenomena during neurogenesis: a selective historical review and progress report. In W.M. Cowan (Ed.), Studies in Developmental Neurobiology: Essays in Honor of Viktor Hamburger, Oxford University Press, New York, 1981, pp. 74–133.

18 Rogers, S.L., McLoon, S.C. and Letourneau, P., Distribution of laminin and fibronectin during early axonal growth in the chick PNS, Soc. Neurosci. Abstr., 10 (1984) 39.

19 Ruoslahti, E., Hayman, E.G., Pierschbacher, M. and Engvall, E., Fibronectin: purification, immunochemical properties, and biological activities, Meth. Enzymol., 82 (1982) 803–831.

20 Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidardt, J.-M. and Martin, G.R., Laminin – a glycoprotein from basement membranes, J. Biol. Chem., 254 (1979) 9933–9937.