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By

Vivian Lynn Steel

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ABSTRACT

PC12 NEURITE OUTGROWTH IN THE ABSENCE OF MICROTUBULES ON EXTRACELLULAR MATRIX

By

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The cytoskeleton has an important structural role in neurite outgrowth of PC12 cells. Actin and microtubules are in a complementary force interaction; actin is under tension which is supported partly by compression of microtubules and partly by attachment to the substratum. We wished to determine the contributions of each component of compressive support during PC12 neurite outgrowth on extracellular matrix (ECM).

NGF-"primed" and "unprimed" PC12 cells exhibited extensive neurite outgrowth during days 1 to 5 on ECM compared to controls. 78% of unprimed and 77% of primed ECM-grown neurites exhibited normal microtubule arrays. Previous work demonstrated Li^+-inhibition of NGF-induced PC12 neurite outgrowth, presumably due to alterations in microtubule-associated proteins required for microtubule assembly. However, we induced neurite outgrowth of both primed and unprimed PC12 cells on ECM in the presence of LiCl: 31% of both primed and unprimed neurites examined lacked microtubules entirely. This is first evidence to date indicating neurite outgrowth in the absence of microtubules.

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INTRODUCTION

BASICS OF NEURONAL GROWTH

The nervous system develops by extending numerous, long cytoplasmic outgrowths, referred to as axons and dendrites, from neuronal cell bodies. The highly asymmetric neuronal cell shape is supported by an internal "cytoskeleton" consisting of actin, neurofilaments, microtubules and a subcellular matrix (Bray and Gilbert, 1981; Ellisman and Porter, 1980; Brady et al., 1984; Hirokawa, 1982; Schnapp and Reese, 1982; Yama 971). The role of the cytoskeleton during neuronal elong has been a subject of interest for many years.

Due to numerous complicating factors involved in the study of nerve growth in vivo, cultured nerve cells have frequently been used as a model for neuronal elongation (Alberts et al., 1983). Many cultured cells extend processes that are difficult to distinguish as axons or dendrites and are thus referred to as "neurites". At the leading edge of the developing nerve cell process (or neurite) is an enlarged structure with an irregular spiky shape called the "growth cone", which is continuously active. It consists of a flattened region from which numerous, long microspikes

alternately extend, adhere to the substratum and then retract back into the growth cone (Landis, 1983). As the growth cone crawls forward it leaves the cell body in place, causing the neurite to grow in length (Alberts et al., 1983).

A process necessary for neurite elongation is the synthesis and assembly of cytoskeletal elements. Current evidence suggests that microtubules are assembled by addition of newly synthesized tubulin subunits at the distal end of the neurite (Bamburg et al., 1986; Hirokawa et al., 1988). New membrane is also probably added at the neurite tip. The importance of specific cytoskeletal components has been determined using drugs which inhibit their polymerization. Cytochalasin B, for example, prevents polymerization of actin into filaments. Addition of cytochalasin B to culture medium inhibits growth cone activity but allows the neurite to remain extended (Heidemann et al., 1985; Joshi et al., 1985; Solomon and Magendantz, 1981; Yamada et al., 1971; Landis, 1983; Bray and Gilbert, 1981). Colchicine (which disrupts microtubules) causes the neurite to retract into the cell body, but does not inhibit growth cone activity or effect new growth cone formation (Daniels, 1972, 1973, 1975; Bray et al., 1978; Heidemann et al., 1985; Jacobs et al., 1986a, Joshi et al., 1985; Solomon and Magendantz, 1981). Thus microtubules appear to stabilize the developing neurite.

Substrate adhesion is another factor important during neurite elongation (Letourneau, 1975, 1979). The growth cone shows a preference for highly adhesive substrata. For example, the negatively charged plasma membrane is strongly attracted to positively charged polymers such as polyornithine and polylysine. Neurons are also strongly adherent to extracellular matrix, a naturally produced complex molecular array (Hay, 1982). It is thought to guide growth cones and control their branching, in vivo (Sanes, 1983; Hay, 1982).

Besides being guided by contact interactions, the growth cone is susceptible to the effects of molecules dissolved in the extracellular fluid. An excellent example is that of nerve growth factor (NGF). Peripheral sensory neurons and many sympathetic neurons require NGF for their survival, as well as to extend neurites (Theonen et al., 1980). NGF has been shown to act directly on the growth cone: a concentration gradient of NGF guides growth cones towards it (Theonen et al., 1980).

PC12 CELLS AND NGF EFFECTS

PC12 is a clonal cell line derived from a rat pheochromocytoma (Greene and Tischler, 1976). When cultured in the presence of NGF for several days, PC12 cells differentiate into cholinergic sympathetic neurons (Theonen et al., 1980). They have thus proven useful as a model system for the study of NGF action on the developing neuron.

The responses of PC12 cells to NGF include both short-term (occurring within seconds or minutes following exposure) and long-term (occurring hours to days after exposure) effects (Greene and Tischler, 1982). The short term effects include the appearance of ruffles on the dorsal cell surface (Connolly et al., 1979), increase in the number of coated pits per unit area of membrane (Connolly et al., 1979, 1980), and increased cell-cell and cell-substrate adhesion (Schubert and Whitlock, 1977; Schubert et al., 1978). In addition to these rapid onset surface alterations, PC12 cells exhibit a significant increase in the rate of transport of amino acids (McGuire and Greene, 1979) and increased phosphorylation of several proteins (Yu et al., 1980; Halegoua and Patrick, 1980).

By 24 hours following exposure to NGF, longer term effects are manifested: the cells "flatten" and exhibit short cytoplasmic spikes. Beyond twenty-four hours, the cells extend long, branching neurites (Greene and Tischler, 1976; Tischler and Greene, 1978) and somatic volume and total protein content per PC12 cell are increased (Greene and Tischler, 1976; Greene and McGuire, 1978). Electron microscopic examination of PC12 neurites exposed to NGF demonstrates the presence of parallel microtubule arrays and clusters of synaptic vesicles (Greene and Tischler, 1976; Tischler and Greene, 1978; Luckenbill-Edds et al., 1979). Following 24-hour exposure, PC12 cells also cease cell division, become electrically excitable, and exhibit

increased synthesis of several proteins (Greene and Tischler, 1976, 1982; McGuire and Greene, 1980).

NGF INDUCTION OF CYTOSKELETAL ELEMENTS

One of the intracellular events that appears to be essential for neurite outgrowth is the formation and extension of microtubule bundles (Daniels, 1973; Luckinbill-Edds et al., 1979; Yamada et al., 1970). PC12 cells exposed to NGF exhibit long, branched neurites containing extensive parallel microtubule bundles, which appear to provide the principal architectural framework for PC12 neurites (Black and Greene, 1982; Greene et al., 1982; Luckinbill-Edds et al., 1979; Tischler and Greene, 1978). In the absence of NGF, PC12 cells are rounded in shape, lack neuritic processes and parallel microtubule arrays (Luckinbill-Edds et al., 1979; Tischler and Greene, 1978). The close temporal relationship between the appearance of compact microtubule bundles and outgrowth of long neurites suggests a causal relationship between these events. Various pharmacological experiments have shown that agents which prevent the formation of microtubule bundles also prevent neurite outgrowth in PC12 cells and other cultured neurons (Greene et al., 1982; Daniels, 1972, 1973; Yamada et al., 1970, 1971).

Tubulin and microtubule associated proteins (MAPs) are the major reactants in microtubule formation. Microtubule associated proteins (MAPs) have been shown to enhance

microtubule assembly and stability. Several studies have shown that the abundance of tubulin and MAPs in PC12 cells increases following treatment with NGF. Greene et al. (1983) first demonstrated that MAP-1 is synthesized and phosphorylated during neurite outgrowth. Drubin et al. (1984, 1985) found that during the first 10 days after exposure to NGF, MAP1 and tau increase 20-fold, while the tubulin pool changes less than 2.5% over this time. Black et al. (1986) demonstrated that long-term NGF treatment increased the level of MAP-2 and influenced post-translational modification of three microtubule associated proteins referred to as the "chartins" in PC12 cells (Mr = 64K, 72K and 80K).

Further investigations using purified microtubule associated proteins (MAPs) from brain or cultured cells indicate that they enhance microtubule stability (Cleveland et al., 1977; Job et al., 1985; Sloboda and Rosenbaum, 1979). MAPs also apparently influence microtubule spacing (Brown and Berlin, 1985; Kim et al., 1979) and are capable of cross-linking microtubules with other cytoskeletal components (Griffith and Pollard, 1982; Hirokawa et al., 1985; LeTerrier et al., 1982; Sattilaro et al., 1981). Thus, the effects on MAPs brought about by NGF appear to contribute to the complex process of microtubule bundle formation and stability.

Black and Greene (1982), observed that neurites grown for several days contained microtubules which were more

resistant to microtubule-depolymerizing drugs than younger neurites. Greene (1984) suggested the enhanced stability could be due to NGF-induction of MAPs and subsequent MAP-microtubule interactions known to occur after at least 3 days of growth. Indeed, NGF-induction of MAP-1 species, MAP-2, chartins and tau MAPs occur in parallel with NGF-induced neurite outgrowth and microtubule stability (Black et al., 1986). Burstein et al. (1985), demonstrated that Li^+ inhibits NGF-induced neurite outgrowth from PC12 cells as well as regeneration of neurites by sympathetic neurons and NGF-primed PC12 cells. Li^+ also specifically inhibits short-term phosphorylation of three proteins (Mr = 64K, 72K and 80K) which have been identified as the chartin MAPs, but not phosphorylation of tubulin or MAP 1.2. This suggests a possible role of phosphorylated chartins in neurite outgrowth.

ADHESION, ECM AND NEURITE OUTGROWTH

Cell growth and differentiation in vivo is the result of a complex balance between cell-cell and cell-substrate interactions. These interactions are mediated by a unique arrangement of macromolecules which constitute the ECM. Extracellular matrix is the material that occupies and maintains spaces between cells. In recent years, several of its components have been characterized (Sanes, 1983) and its role in neuronal development has been investigated (Hawrot, 1980; Loring et al., 1982; Akers et al., 1981; Baron Van-

Evercooren et al., 1982a,b; Carbonetto et al., 1983; Rogers et al., 1983; Edgar et al., 1984; Lander et al., 1982; Letourneau, 1975; Carbonetto, 1984). These and other studies have established that an important function of ECM is that of cell adhesion: this is a step thought to be necessary for neuronal differentiation, outgrowth and guidance to occur.

Effects of the culture substratum on neurite outgrowth are well documented. Letourneau (1975) correlated increased neuron-substratum adhesion with rapid and significant neurite outgrowth. On more adhesive substrates, the growth cone flattens out and spreads further than on poorly adhesive substrates (Letourneau, 1979). Davis et al. (1985), found that dissociated E8 chick ciliary ganglion neurons exhibited enhanced neurite initiation time and total neuritic output per neuron on laminin compared to other surfaces. Additionally, Bray et al. (1987) found that sensory neurons dissociated from lumbar dorsal root ganglia of chick embryos exhibited enhanced growth on surfaces prepared with laminin or conditioned medium compared to glass or collagen surfaces.

Laminin and fibronectin, predominant glycoproteins in ECM have been shown to enhance neurite elongation of both central and peripheral neurons (Akers et al., 1981; Baron Van-Evercooren et al., 1982a; Faivre-Bauman et al., 1984; Manthorpe et al., 1983; Rogers et al., 1983). Regional distributions of these molecules suggest their role in determination of preferred peripheral nervous system

neuronal pathways (Rogers et al., 1986). Although several experiments have demonstrated that laminin is the preferred substrate for neurite elongation (Smalheiser et al., 1984; Hammarback et al., 1985; Gundersen, 1987), direct adhesion measurements show that laminin decreases overall adhesion, arguing against the importance of adhesion during neurite elongation (Gundersen, 1987).

The absence of contaminating nonneuronal cells also renders the PC12 cell line useful for investigations of neuronal adhesion mechanisms (Luduena, 1973). NGF has been shown to enhance both cell-cell and cell-substratum adhesion in PC12 cells (Schubert and Whitlock, 1977; Chandler and Herschman, 1980; Fujii et al., 1982). Schubert (1979) suggested that neurite outgrowth could be caused specifically by increased cell adhesiveness. However, when plated in the presence of epidermal growth factor (EGF), although PC12 cells exhibit increased cell-substratum attachment, they lack neurite outgrowth (Chandler and Herschmann, 1980), demonstrating that adhesion alone is not sufficient to explain the neurite outgrowth-promoting aspects of NGF.

Turner et al. (1987) found that PC12 cells extended neurites on laminin, native collagens I/III, II and IV and on denatured collagen IV, but not on denatured collagens I/III or II, nerve growth factor or wheat germ agglutinin.

CYTOSKELETAL FUNCTIONS; COMPLEMENTARY FORCE MODEL FOR NEURITE OUTGROWTH

The cytoskeleton is thought to provide the mechanical basis for cell shape, (Alberts et al., 1983), including the highly asymmetric structure of neuronal cells (Landis, 1983). The two most important proteins present in the cytoskeleton are actin and tubulin. Specific intracellular mechanisms exist which control the assembly of these and accessory proteins from soluble protein pools in the cytoplasm into an actin network and microtubules (Alberts et al., 1983). Actin and microtubules are crucial to various cell functions, such as cell division, motility and cytoplasmic extension (Alberts et al., 1983).

Numerous studies on cultured neurons have indicated a clear cut "division of labor" of microtubules and actin filaments during growth cone motility and axonal elongation (Landis, 1983; Yamada et al., 1970). Drugs that disrupt microtubules (colchicine and colcemid) cause neurite retraction, but have no effect on growth cone motility (Daniels, 1972, 1973 and 1975; Bray et al., 1978; Heidemann et al., 1985; Jacobs et al., 1986b; Joshi et al., 1985; Solomon and Magendantz, 1981). Cytochalasins act by inhibiting polymerization of actin filaments (Flanagin and Lin, 1980; Brown and Spudich, 1979). They have been shown to stabilize the neurite to retraction, but also to inhibit filopodial contractility (Bray et al., 1978; Heidemann et al., 1985; Joshi et al., 1985; Solomon and Magendantz, 1981;

Yamada et al., 1971; Bray and Gilbert, 1981; Landis, 1983). This contractility is presumably responsible for tension development in cultured neurites, which influences growth cone motility (Bray, 1979). Additionally, drugs that augment microtubule assembly stabilize neurites to retraction and sometimes cause extension, while drugs that augment actin assembly cause retraction (Corvaja et al., 1982; Joshi et al., 1985; Letourneau et al., 1987; Solomon and Magendantz, 1981).

Several lines of evidence have demonstrated the importance of mechanical force during axonal elongation. Bray (1979, 1984) originally demonstrated that neurites are under tension and induced neurite outgrowth using experimentally applied tension. Heidemann et al.(1985) observed neurite retraction in response to microtubule depolymerization, consistent with microtubules being under compression. Joshi et al. (1985), used retraction as an assay for neurite tension and drugs to manipulate various cytoskeletal elements. They demonstrated that microtubules in PC12 neurites are under compression, supporting tension in the actin network: disruption of the actin network inhibited neurite retraction, while actin polymerization facilitated retraction. Depolymerization of microtubules resulted in neurite retraction, while microtubule stabilization did the opposite. Recently, direct tension measurements made in PC12 neurites using force calibrated needles supported these observations (Dennerll et al.,

1988). This evidence supports the hypothesis that actin and microtubules are in a complementary force interaction: the actin network is under tension, which is supported in part by compression of microtubules. This complementary force interaction is similar to the force balance among structural elements that underlies the "tensegrity" (tensioned integrity) architectural principle, first developed and named by Buckminster Fuller (1961).

We have used the PC12 cell line as a model system to examine the morphological and cytoskeletal changes that occur in cells grown on various substrata (extracellular matrix, polylysine) and in the presence of various molecules (nerve growth factor, lithium) dissolved in the extracellular fluid.

INTRODUCTION

The importance of the cytoskeleton to neuronal structure and axonal elongation is well documented (Yamada et al., 1970; Bray and Gilbert, 1981). Most evidence suggests that axonal elongation of cultured neurons occurs by the motility and mass addition at the growth cone (Landis, 1983; Goldburg and Burmeister, 1986; Bamburg et al., 1987). The motility of the growth cone and its ability to advance is widely regarded to depend upon actin function (Yamada et al., 1971; Landis, 1983; Forscher and Smith, 1988). Many experiments had shown that addition of cytochalasin D to elongating neurons inhibited growth cone motility and subsequent elongation (Yamada et al., 1971; Forscher and Smith, 1988; Bray 1984, 1985; Joshi et al., 1985; Letourneau et al., 1987; Solomon and Magenstanz, 1981). However, Marsh and Letourneau (1984) demonstrated neurite outgrowth by cultured chick dorsal root ganglion cells in the presence of cytochalasin on highly adhesive substrata. The neurites observed were abnormal, being markedly curved in contrast to the straight growth typical of neurites cultured without cytochalasin.

We have recently studied the structural roles of the cytoskeleton in neurite outgrowth of PC12 cells. We find that actin and microtubules of PC12 neurites are in a complementary force interaction; actin is under tension which is supported in part by compression of microtubules

(Joshi et al., 1985; Dennerll et al., 1988). In principle, neurite elongation could occur through an actin-based tensile mechanism (pulling) or through a microtubule-based compressive mechanism (Buxbaum and Heidemann, 1988). Indeed, whether the growth cone is pulling or pushing has been a source of recent controversy (Letourneau et al., 1987; Bray and White, 1988; Goldburg and Burmeister, 1986). Our data (Dennerll et al., 1988) suggests that neurite elongtion in the presence of cytochalasin (Marsh and Letourneau, 1984) or cytochalasin and taxol (Spero and Roisen, 1985; Letourneau et al., 1987) occurs under net compression. Curved growth is expected because pushing within the flexible neurite is like pushing on a rope. Under more normal growth conditions for cultured neurons, this complementary force hypothesis suggests that microtubules would need to be rather stable in order to support the tension of the actin network because compression destabilizes microtubules, tending to cause disassembly (Hill and Kirschner, 1982; Buxbaum and Heidemann, 1988). Alterations in microtubule assembly accompanying NGF-induced neurite outgrowth of PC12 cells is consistent with the need for increased microtubule stability during neurite elongation; PC12 neurite outgrowth in response to NGF is accompanied by an increase in microtubule stability to anti-microtubule drugs (Heidemann et al., 1985; Black et al., 1986), an increase in tubulin synthesis (Drubin et al., 1985; Black et al., 1986) and a marked increase in synthesis of assembly-promoting microtubule

associated proteins (Drubin et al., 1985; Black et al., 1986; Greene et al., 1983; Peng et al., 1985; Magendantz and Solomon, 1985).

We found the reports of Vlodavsky et al. (1982) and Fujii et al. (1982) particularly interesting in respect to our complementary force model and the role of microtubules in neurite elongation. These workers showed that PC12 cells grown on extracellular matrix (ECM) in the absence of NGF induced rapid but transient neurite outgrowth. They concluded that ECM mimics some of the activity of NGF. We were interested in the role of microtubules in this rapid neurite outgrowth. However, neurite outgrowth on ECM appears to be too rapid to require the transcriptionally-dependent changes in microtubule stability that accompany NGF-induced neurite outgrowth. Does the increased adhesion of PC12 cells to ECM relative to other substrates (Sanes, 1983) permit all of the putative compressive support to be provided by attachment to the dish? If so, are microtubules now expendable for neuronal growth? We have extended the work of Vlodavsky et al. (1982) and Fujii et al. (1982) with particular emphasis on the occurrence and need for microtubules in ECM-induced outgrowth of PC12 neurites.

MATERIALS AND METHODS

PC12 cells were cultured as previously described (Heidemann et al., 1985). "Primed" cells (Burstein and Greene, 1978) were grown for 6 days in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% horse serum, 5% fetal bovine serum and 50 ng/ml 7s NGF. Cultures were maintained at 37° C in a humidified atmosphere containing 10% CO_2. "Unprimed" cells were grown under the same conditions excluding NGF. At the beginning of an experiment, cells (primed or unprimed) were triturated from the surface of the dish and replated at a density of 5×10^4 onto 60 mm tissue culture dishes with varying substratum treatments. Preliminary experiments showed that at this density neurite outgrowth was maximized on extracellular matrix. One experimental group was grown on tissue culture dishes (Corning Medical and Scientific Corporation, Medfield, MA) treated with 1 mg/ml polylysine for 30 minutes and then rinsed twice with sterile distilled water. Another group was grown on commercially obtained dishes with extracellular matrix coated culture surfaces (Extracell dishes, coated with extracellular matrix produced from corneal endothelial cells; Accurate Chemical and Scientific Corporation, Westbury, NY). All cells were maintained in RPMI-1640 containing horse and fetal bovine sera as above in the presence or absence of additional experimental

variables, 50 ng/ml NGF and 10 mM LiCl, as described in Results.

Neurite outgrowth was assessed quantitatively by an assay similar to that previously described (Heidemann et al., 1985). On day 1 of the experiment, cultures were examined using an inverted phase microscope and four 1 mm circles were marked on the bottom of culture dishes. On days 1-5, the total number of neurites and cell bodies for a given experimental treatment were counted from photographs that surveyed the circled regions.

Cells were fixed, processed and observed by transmission electron microscopy as previously described (Baas and Heidemann, 1986).

RESULTS

We confirmed the results of Vlodavsky et al. (1982) and Fujii et al. (1982) that ECM stimulates a rapid, but temporary, outgrowth of PC12 neurites in the absence of nerve growth factor (NGF). Table 1 summarizes experiments comparing neurite outgrowth of PC12 cells treated with nerve growth factor plated on polylysine with the neurite outgrowth of PC12 on extracellular matrix (ECM) without nerve growth factor. An outgrowth extending from the cell body was specified as a neurite if it was greater than two cell bodies in length; these were more selective criteria than used in past studies (Heidemann et al., 1985). Data collection in some experiments was conducted only between days 3 to 5 due to culture contamination. The quantitative decreased number of cells observed on Day 4 were due to photographic difficulties during one experiment. On ECM, the apparent peak of neurite outgrowth occurred on day 3 when there were nearly as many neurites as cell bodies, compared to about 20% frequency of neurites: cell body on nerve growth factor stimulated cells. We were surprised by the high, but transient, neurite frequency for NGF-treated cells on day 2; this was a combined value of four experiments which showed some numerical variability on this day.

We examined by transmission electron microscopy five neurites grown on ECM for three or four days. Consistent with the results of Fujii et al. (1982), 78% had a

Table 1: Percentage of the number of neurites per number of cell bodies of "unprimed" PC12 cells grown on polylysine in the presence of nerve growth factor (NGF) or on extracellular matrix in the absence of NGF for five days.

Day	Polylysine w/ NGF		Extracellular matrix w/o NGF	
1	34/474	7%	232/637	36%
2	201/445	45%	333/660	50%
3	170/1091	16%	751/769	98%
4	74/608	12%	345/568	61%
5	226/1276	18%	232/316	73%

microtubule array (Table 2, Fig. 1) that we judged as being within the normal density range based on our previous ultrastructural observations of NGF-induced neurites (Joshi et al., 1985). We were able to observe the terminal of seven neurites and found that two of these contained "loops" of microtubules (Fig. 2), similar to those previously observed by Tsui et al. (1984) and Letourneau and Ressler (1984).

PC12 cells that have previously been exposed to NGF for several days are able to regrow significant numbers of neurites within a day (Burstein and Greene, 1978). This regrowth of "primed" PC12 cells on collagen or polylysine treated surfaces requires the presence of NGF (Burstein et al., 1978). We wished to determine whether ECM could substitute for NGF in the rapid regrowth of primed PC12 cells. Replating primed PC12 cells onto ECM in the absence of NGF produced neurite outgrowth, in marked contrast to primed cells plated on polylysine without NGF (Table 3). In many experiments, the neurites stimulated by ECM were the longest and most rapidly growing we have seen for PC12, even after only one day of outgrowth (Fig. 3). These neurites contained microtubule arrays of apparently normal density: 37 of the 48 neurites examined (77%) contained normal microtubule arrays, similar to those of unprimed PC12 cells grown on ECM (Table 2). Of the 39 primed nerve terminals examined ultrastructurally, 44% of the terminals on ECM contained "loops" of microtubules, similar to the loops observed in unprimed cells (Tsui et al., 1984; Letourneau

Table 2: Percentage of day 3 or 4 neurites containing microtubules from "primed" and "unprimed" PC12 cells grown on extracellular matrix in the presence or absence of LiCl.

Treatment	+	-	o
"unprimed" on ECM (n=5)	78%	22%	0%
"primed" on ECM (n=48)	77%	19%	4%
"unprimed" on ECM + LiCl (n=29)	45%	24%	31%
"primed" on ECM + LiCl (n=31)	36%	33%	31%

+ = presence of "average" number of microtubules

- = negligible number of microtubules

o = absence of microtubules

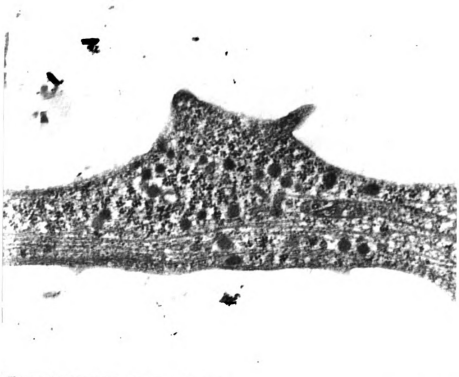

Figure 1. Electron micrograph of a "normal" microtubule array in an "unprimed" PC12 neurite grown on extracellular matrix.

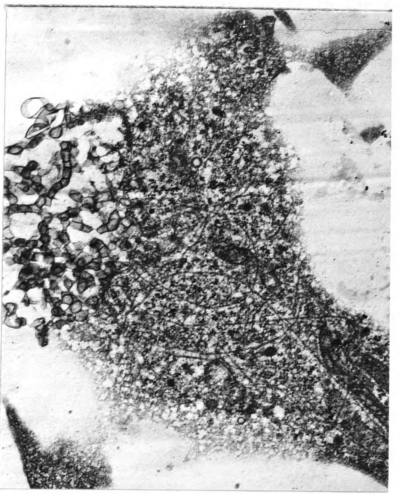

Figure 2. Electron micrograph of microtubule "loops" in the nerve terminal of an unprimed PC12 neurite grown on extracellular matrix.

Table 3: Percentage of the number of neurites per number of cell bodies of "primed" PC12 cells grown on polylysine or on extracellular matrix in the absence of NGF for five days.

Day	Polylysine		Extracellular matrix	
1	0/73	0%	53/109	49%
2	0/76	0%	47/152	31%
3	0/123	0%	91/215	42%
4	0/129	0%	54/208	26%
5	0/105	0%	47/204	23%

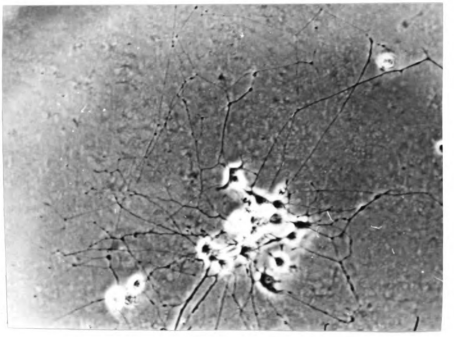

Figure 3. Day 1 neurite outgrowth of "primed" PC12 cells plated onto extracellular matrix in the absence of nerve growth factor.

and Ressler, 1984; Fig. 4). Several nerve terminals also contained a large number of ribosomes (Fig. 4), similar to those described by Fujii et al. (1982).

LiCl has been shown to block neurite outgrowth, apparently by its ability to selectively inhibit phosphorylation of three microtubule associated proteins (MAPs) identified as the "chartins", which have been shown to have a role in neurite outgrowth and appear to stimulate MT assembly when phosphorylated (Burstein et al., 1985). Addition of 10 uM LiCl to medium containing NGF suppressed neurite outgrowth on polylysine treated surfaces; less than 10% of cell bodies produced neurites during five days of observation (data not shown), as previously reported (Burstein et al., 1985). However, both primed and unprimed PC12 cells plated in the presence of this Li^+ concentration showed significant neurite outgrowth (Table 4, Fig. 5), although there was less neurite outgrowth than in Li+-free conditions (Tables 1 and 3; Fig. 3).

Electron microscopic examination of Li^+-treated neurites of unprimed PC12 cells grown on ECM revealed that only 45% of the neurites examined contained normal microtubule arrays compared to 78% in control ECM neurites. Additionally 9 of 29 Li^+-treated, unprimed cells were substantially devoid of microtubules (Table 2; Fig. 6). Less than 5% of neurites grown in Li^+-free conditions were similarly devoid of microtubules (Table 2). Similarly, primed PC12 cells exhibited a significant decrease in microtubules. Ten of 31

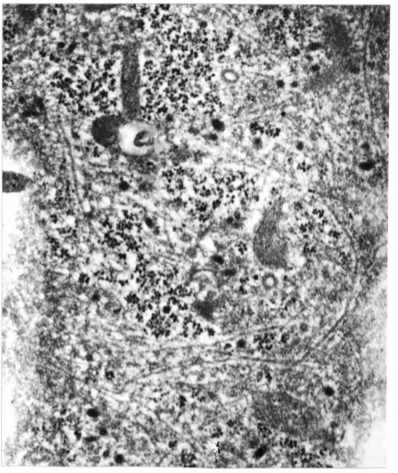

Figure 4. Electron micrograph of microtubule "loops" in the nerve terminal of a "primed" PC12 neurite grown on extracellular matrix.

Table 4. Percentage of the number of neurites per number of cell bodies of "primed" and "unprimed" PC12 cells plated on extracellular matrix in the presence of LiCl for five days.

Day	"unprimed"		"primed"	
1	127/567	22%	10/67	15%
2	239/560	43%	31/111	28%
3	200/598	33%	28/105	27%
4	162/300	54%	27/69	39%
5	39/238	17%	15/66	22%

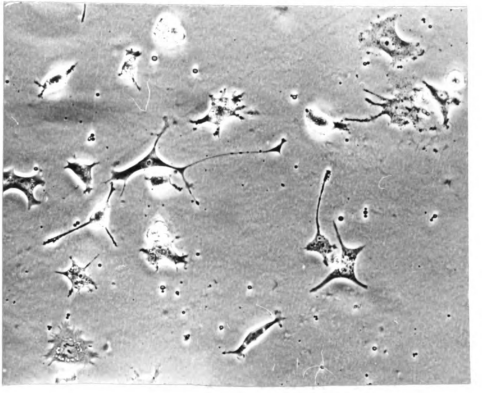

Figure 5. Day 4 neurite outgrowth by "unprimed" PC12 cells grown on extracellular matrix in the presence of LiCl.

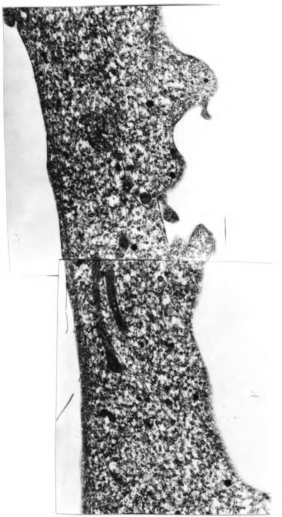

Figure 6. Electron micrograph of an "unprimed" PC12 neurite grown on extracellular matrix in the presence of LiCl.

neurites examined were substantially devoid of microtubules. As in unprimed cells, only short, ambiguous tubular elements were observed (Fig. 7; Table 2).

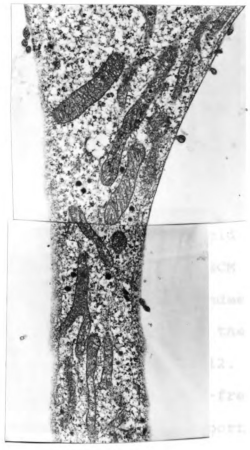

Figure 7. Electron micrograph of a "primed" PC12 neurite grown on extracellular matrix in the presence of LiCl.

DISCUSSION

Our results confirm and extend those of Vlodavsky et al. (1982) and Fujii et al. (1982) that extracellular matrix (ECM) substitutes in some way for the action of nerve growth factor (NGF) in the initial stages of neurite outgrowth. PC12 cells on ECM that had not been previously exposed to NGF showed a peak of neurite outgrowth on day 3 after plating; outgrowth declining rapidly thereafter. It is postulated that the delay in neurite outgrowth following exposure to NGF is due to an RNA transcription-dependent process called "priming" (Burstein et al., 1978). Plating NGF-primed cells onto ECM promotes rapid neurite outgrowth, again suggesting the substitution of ECM for the normally required continued presence of NGF. Indeed, outgrowth of neurites from primed cells on ECM was the most rapid and extensive that we have observed in PC12. Plating primed cells onto polylysine in the same NGF-free medium produced no neurite outgrowth as previously reported (Burstein et al., 1985).

Because PC12 neurite outgrowth is closely correlated with microtubule assembly and stability (Black et al., 1986; Joshi et al., 1985; Dennerll et al., 1988), we postulated that the ECM effect might include an effect on promoting microtubule polymerization, organization and/or stability. Using electron microscopy, we found that 80% of the neurites of NGF or ECM grown cells, both primed and unprimed,

contained normally extensive arrays of microtubules. We take this to indicate that microtubule assembly on ECM is as least as active as with NGF. Additionally, a significant fraction of ECM neurite terminals contained unusual microtubule "loops". Such loops were not observed in NGF-induced neurites in this nor in previous ultrastructural studies of PC12 (Joshi et al., 1985; Heidemann et al., 1985). Similar loops appear, however, in chick sensory neurons exposed to the MT-assembly-promoter, taxol (Letourneau and Ressler, 1974) and in embryonic chick retinal neurons (Tsui et al., 1984). Like these workers, we suggest microtubule loops occur due to increased microtubule polymerization.

To our surprise, we have found that microtubule assembly is not required for neurite outgrowth: LiCl, which has been shown to inhibit NGF-induced neurite outgrowth of PC12 cells, presumably by inhibiting phosphorylation of certain microtubule associated proteins, does not prevent neurite outgrowth on ECM. Approximately 30% of the neurites grown on ECM in the presence of LiCl appeared completely devoid of microtubules. To our knowledge, this is the first report of neurite outgrowth in the absence of microtubules.

Mechanical measurements on PC12 neurites indicate that microtubules provide compressive support to the tensile neurites (Dennerll et al., 1988). Thermodynamics predicts that compression will destabilize microtubules and promote their disassembly (Hill and Kirschner, 1982; Buxbaum and

Heidemann, 1988). Experimentally, increased compression of PC12 neurites resulted in an increase in the soluble tubulin pool (Dennerll et al., 1988). We previously postulated that the increased stability of microtubules accompanying neurite outgrowth is necessary to support the compressive load (Heidemann et al., 1985; Joshi et al., 1985). The data reported here suggest to us that growth on ECM shifts the compressive support from internal microtubules to the external substratum. Even after inhibition of microtubule assembly by LiCl, we believe that the significant adhesivity of ECM allows tension present in the actin network to be completely supported externally. Similarly, this arguement can be used to explain the presence of microtubule loops in PC12 neurites grown on ECM: If tension in the actin network is supported externally on ECM, decreased microtubule compression would shift the microtubule assembly equilibrium reaction in favor of tubulin polymerization, facilitating microtubule assembly and the formation of loop structures.

LIST OF REFERENCES

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Akers, R.M., D.F. Mosher and J.E. Lilien, Promotion of retinal neurite outgrowth by substrate-bound fibronectin. Dev Biol. 86:179-188, 1981.

Alberts, B.M., D. Bray, J. Lewis, M. Raff, K. Roberts and J.D. Watson. Molecular Biology of the Cell. Garland Publishing Inc., N.Y., pp. 549-609; 1070-1077, 1983.

Baas, P.W. and S.R. Heidemann, Microtubule reassembly from nucleating fragments during the regrowth of amputated neurites. J. Cell Biol. 103:917-927, 1986.

Bamburg, J.R., D. Bray and K. Chapman, Assembly of microtubules at the tip of growing axons. Nature 321: 788-790, 1986.

Baron-Van Evercooren, A., H.K. Kleinman, S. Ohno, P. Marangos, J.P. Schwartz, and M.E. Dubois-Dalcq, Nerve growth factor, laminin and fibronectin promote neurite growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8:179-183, 1982a.

Baron-Van Evercooren, A., H.K. Kleinman, H.E.J. Seppa, B. Rentier and B. Dubois-Dalcq, Fibronectin promotes rat schwann cell growth and motility. J. Cell Biol. 93:211-216, 1982b.

Black, M.M., J.M. Aletta and L.A. Greene, Regulation of microtubule composition and stability during nerve growth factor (NGF)-promoted neurite outgrowth. J. Cell Biol. 103:545-557, 1986.

Black, M.M. and L.A. Greene, Changes in the colchicine susceptibility of microtubules associated with neurite outgrowth: Studies with nerve growth factor-responsive PC12 pheochromocytoma cells. J. Cell Biol. 95:379-386, 1982.

Brady, S.T., M. Tytell and R.J. Lasek, Axonal tubulin and axonal microtubules: biochemical evidence for

cold stability. J. Cell Biol. 99:1716-1724, 1984.

Bray, D., Axonal growth in response to experimentally applied tension. Dev. Biol. 102:379-389, 1984.

Bray, D., Mechanical tension produced by nerve cells in tissue culture. J Cell Sci. 37:391-410, 1979.

Bray, D., M.B. Bunge and K. Chapman, Geometry of isolated sensory neurons in culture. Exp. Cell Res. 168:127-137, 1987.

Bray, D. and D. Gilbert, Cytoskeletal elements in neurons. Ann Rev. Neurosci. 4:505-523, 1981.

Bray, D., C. Thomas and G. Shaw, Growth cone formation in cultures of sensory neurons. Proc. Natl. Acad. Sci., 75:5226-5229, 1978.

Bray, D. and J.G. White, Cortical flow in animal cells. Science. 239:883-888, 1988.

Brown, P.A. and R.D. Berlin, The packing volume of sedimented microtubules: regulation and potential relationship to a potential intracellular matrix. J. Cell Biol. 101:1492-1500, 1985.

Brown, S.S. and J.A. Spudich, Cytochalasin inhibits the rate of of elongation of actin filament fragments. J. Cell Biol. 83:657-662, 1979.

Burstein, D.E. and L.A. Greene, Evidence for both RNA-synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. Proc. Nat. Acad. Sci. 75:6059-6063, 1978.

Burstein, D.E., P.J. Seeley and L.A. Greene, Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. J. Cell Biol. 101:862-870, 1985.

Buxbaum, R. and S.R. Heidemann, A thermodynamic model for microtubule assembly during axonal growth. J. Theor. Biol. 134:379-390, 1988.

Carbonetto, S., The extracellular matrix of the nervous system. Trends Neurosci. 7:382-387, 1984.

Carbonetto, S., M.M. Gruver and D.C. Turner, Nerve fiber growth in culture on fibronectin, collagen and glycosaminoglycan substrates. J. Neurosci.

3:2324-2335, 1983.

Chandler, C.E. and H.R. Herschmann, Tumor promoter modulation of epidermal growth factor and nerve growth factor-induced adhesion and growth factor binding of PC12 pheochromocytoma cells. J. Cell Physiol. 105:275-285, 1980.

Cleveland, D.W., S.Y. Hwo and M.W. Kirschner, Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. J. Mol. Biol. 116:227-247, 1977.

Connolly, J.L. and L.A. Greene, Pit formation and rapid changes in surface morphology of sympathetic neurons in response to nerve growth factor. J. Cell Biol. 90:176-180, 1980.

Connolly, J.L, L.A. Greene, R.R. Viscarello and W.D. Wiley, Rapid, sequential changes in surface morphology of PC12 pheochromocytoma cells in response to nerve growth factor. J. Cell Biol. 82:820-827, 1979.

Corvaja, N., A. DiLuzio, S. Biocca, A. Cattaneo and P. Callisano, Morphological and ultrastructural changes in PC12 pheochromocytoma cells induced by combined treatment with taxol and NGF. Exp. Cell Res. 142:385-395, 1982.

Daniels, M.P., Colchicine inhibition of nerve fiber formation in vitro. J. Cell Biol., 53:164-176, 1972.

Daniels, M.P., Fine structural changes in neurons and nerve fibers associated with colchicine inhibition of nerve fiber formation in vitro. J. Cell Biol. 58:463-470, 1973.

Daniels, M.P.,The role of microtubules in the growth and stabilization of nerve fibers. Ann. NY Acad. Sci. 253:535-544, 1975.

Davis, G.E., M. Manthorpe and S. Varon, Parameters of neuritic growth from ciliary ganglion neurons in vitro- influence of laminin, schwannoma polyornithine-binding neurite promoting factor and ciliary neurotrophic factor. Dev. Brain Res. 17:75-84, 1985.

Dennerll, T.J., H.C. Joshi, V.L. Steel, R.E. Buxbaum and S.R. Heidemann, Tension and compression in the cytoskeleton of PC-12 neurites II: Quantitative measurements. J. Cell Biol. 107: 665-674, 1988.

Drubin, D., S. Feinstein, E. Shooter and M. Kirschner, Nerve growth factor-induced outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. J. Cell Biol. 101:1799-1807, 1985.

Drubin, D., M. Kirschner and S. Feinstein, Microtubule-associated tau protein induction by nerve growth factor during neurite outgrowth in PC-12 cells. In, Borisy, G.G., D. Cleveland and D.B. Murphy eds. Molecular biology of the cytoskeleton. Cold Spring Harbor, New York: Cold Spring Harbor lab, 512 pp., 1984.

Edgar, D., R. Timpl and H. Thoenen, The heparin-binding domain of laminin is responsible for for its effects on neurite outgrowth and neuronal survival. EMBO J. 3:1463-1468, 1984.

Ellisman, M.H. and K.R. Porter, Microtrabecular structure of the axoplasmic matrix: visualization of cross-linking structures and their distribution. J. Cell Biol. 87:464-479, 1980.

Faivre-Bauman, A., J. Putmirat, J. Loudes, A. Barret and A. Tixier-Vidal, Laminin promotes attachment and neurite elongation of fetal hypothalamic neurons grown in serum-free culture medium. Neurosci. Lett. 44:83-89, 1984.

Flanagan, M.D. and S. Lin, Cytochalasins block actin filament elongation by binding to high-affinity sites associated with F-actin. J. Biol. Chem. 255:835-838, 1980.

Forscher, P. and S.J. Smith, Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J. Cell Biol. 107:1505-1516, 1988.

Fujii, D.K., S.L. Massoglia, N. Savion, and D. Gospodarowicz, Neurite outgrowth and protein synthesis by PC-12 cells as a function of substratum and nerve growth factor. J. Neurosci. 2:1157-1175, 1982.

Fuller, R.B., Tensegrity. Portfolio and Artnews Annual. 4:112-127, 1961.

Goldburg, D.J. and D.W. Burmeister, Stages in axon formation: observation of growth of Aplysia axons in culture using video-enhanced contrast-

differential interference contrast microscopy. J. Cell Biol. 103: 1921-1931, 1986.

Greene, L.A., The importance of both early and delayed responses in the biological actions of nerve growth-factor. Trends Neur. 7(3):91-94, 1984.

Greene, L.A., D.E. Burstein and M.M. Black, The role of transcription dependent priming in nerve growth factor promoted neurite outgrowth. Dev. Biol. 91:305-316, 1982.

Greene, L.A., R.K. Liem and M.L. Shelanski, Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. J. Cell Biol., 96:76-83, 1983.

Greene L.A. and J.C. McGuire, Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. Nature. 276:191-194, 1978.

Greene, L.A. and A.S. Tischler, Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. 73:2424-2428, 1976.

Greene, L.A. and A.S. Tischler, PC12 pheochromocytoma cultures in neurobiological research. Adv. Cell. Neurobiol. 3:373-414, 1982.

Griffith, H. and T.D. Pollard, The interaction of actin filaments with microtubules and microtubule-associated proteins. J. Biol. Chem. 27:9143-9151, 1982.

Gundersen, R.W., Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin in vitro. Dev. Biol. 121:423-431, 1987.

Halegoua, S. and Patrick, J., Nerve growth factor mediates phosphorylation of specific proteins. Cell. 22:571-581, 1980.

Hammarback, J.A., S.L. Palm, L.T. Furcht, and P.C. Letourneau, Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. J. Neurosci. Res. 13:213-222, 1985.

Hawrot, E., Cultured sympathetic neurons: effects of cell-derived and synthetic substrata on growth and development. Dev. Biol. 74:136-151, 1980.

Hay, E.D. Cell Biology of Extracellular Matrix. Plenum Press, N.Y., 417 pp., 1982.

Heidemann S.R., H.C. Joshi, A. Schechter, J.R. Fletcher and M. Bothwell, Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC-12 cells. J. Cell Biol. 100:916-927, 1985.

Hill, T.L. and M.W. Kirschner, Bioenergetics and kinetics of microtubule and actin filament assembly-disassembly. Int. Rev. Cytol. 78:1-125, 1982.

Hirokawa, N., Cross-linker system between neurofilaments, microtubules and membranous organelles in frog axons revealed by the quick- freeze deep-etching method. J. Cell Biol. 94:124-142, 1982.

Hirokawa, N., G.S. Bloom and R.B. Vallee, Cytoskeletal architecture and immunocytochemical localization of microtubule-associated proteins in regions of axons associated with rapid axonal transport: the B,B-iminodipropionnitrile-intoxicated axon as a model system. J. Cell Biol. 101:227-239, 1985.

Jacobs, J.R. and J.K. Stevens, Changes in the organization of the neuritic cytoskeleton during nerve growth factor-activated differentiation of PC-12 cells: a serial electron microscopic study of the development and control of neuite shape. J. Cell Biol. 103:895-906, 1986a.

Jacobs, J.R. and J.K. Stevens, Experimental modification of PC12 neurite shape with the microtubule-depolymerizing drug nocadozole: a serial electron microscopic study of neurite shape control. J. Cell Biol. 103:907-915, 1986b.

Job, D., M. Pabion, and R.L. Margolis, Generation of microtubule assembly subclasses by microtubule-associated proteins: implications for the microtubule "dynamic instability" model. J. Cell Biol. 101:1680-1689, 1985.

Joshi, H.C., D. Chu, R.E. Buxbaum and S.R. Heidemann, Tension and compression in the cytoskeleton of PC12 neurites. J. Cell Biol. 101:697-705, 1985.

Kim, H., L.I. Binder and J.R. Rosenbaum, The periodic association of MAP-2 with brain microtubules in vitro. J. Cell Biol. 80:226-276, 1979.

Lander, A.D., D.K. Fujii, D. Gospodarowitz and L.F. Reichardt, Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparin sulfate proteoglycan. J. Cell Biol. 94:574-585, 1982.

Landis, S.C., Neuronal growth cones. Annu. Rev. Physiol. 45:567-580, 1983.

LeTerrier, J.F., R.K.H. Liem and M. Shelanski, Interactions between neurofilament and microtubule-associated proteins: a possible mechanism for intraorganelle bridging. J. Cell Biol. 95:982-986, 1982.

Letourneau, P.C., Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. Dev. Biol. 44:77-91, 1975.

Letourneau, P.C., Cell-substratum adhesion of neurite growth cones and its role in neurite elongation. Exp. Cell Res. 124:127-138, 1979.

Letourneau, P.C. and A.H. Ressler, Inhibition of neurite initiation and growth by taxol. J. Cell Biol. 98:1355-1362, 1984.

Letourneau, P.C., T.A. Shattuck, and A.H. Ressler, "Pull" and "push" in neurite elongation: observations on the effects of different concentrations of cytochalsin B and taxol. Cell Motil. Cytoskeleton 8:193-209, 1987.

Loring, J., B. Glimelius and A.J. Weston, Extracellular matrix materials influence quail neural crest cell differentiation in vitro. Dev. Biol. 90:165-174, 1982.

Luckenbill-Edds L., C. Van Horn and L.A. Greene, Fine structure of initial outgrowth of processes induced in a pheochromocytoma cell line (PC12) by nerve growth factor. J. Neurocyt. 8:493-511, 1979.

Luduena, M.A., The growth of spinal ganglion neurons in serum-free medium. Dev. Biol. 33:470-476, 1973.

Magendantz, M and F. Solomon, Analyzing the components of microtubules: antibodies against chartins, associated proteins from cultured cells. Proc. Natl. Acad. Sci. 82:6581-6585, 1985.

Manthorpe, M., E. Engvali, E. Ruoshlati, F.M. Longo, G.E. Davis and S. Varon, Laminin promotes neuritic

regeneration from cultured central and peripheral neurons. J. Cell Biol. 97:1882-1890, 1983.

Marsh, L. and P.C. Letourneau, Growth of neurites without filopodial or lamellipodial activity in the presence of Cytochalasin B. J. Cell Biol. 99:2041-2047, 1984.

Mason, C., How do growth cones grow?, Trends in Neurosci. 8:304-306, 1985.

McGuire, J.C and L.A. Greene, Rapid stimulation by nerve growth factor of amino acid uptake by clonal PC12 pheochromocytoma cells. J. Biol. Chem. 254:3362-3367, 1979.

McGuire, J.C. and L.A. Greene, Nerve growth factor stimulation of specific protein synthesis by rat PC12 pheochromocytoma cells. Neuroscience. 5:179-189, 1980.

Peng, I., L.I Binder and M.M. Black, Cultured neurons contain a varity of microtubule-associated proteins, Brain Res. 361:200-211, 1985.

Rogers, S.L., K.L. Edson, P.C. Letourneau, and S.C. McLoon, Distribution of laminin in the developing peripheral nervous system of the chick. Dev. Biol. 113:429-435, 1986.

Rogers, S.L., P.C. Letourneau, S.L. Palm, J. McCarthy and T. Furcht, Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. 98:212-220, 1983.

Sanes, J.R., Roles of extracellular matrix in neural development, Annu. Rev. Physiol. 45:581-600, 1983.

Schnapp, B.J. and T.S. Reese, Cytoplasmic structure in rapid frozen axons. J. Cell Biol. 94:667-679, 1982.

Schubert, D., Early events after the the interaction of NGF with sympathetic nerve cells. Trends Neurosci. 2:17-23, 1979.

Schubert, D., M. Lacorbiere, C. Whitlock and W. Stallcup, Alterations in the surface properties of cells responsive to nerve growth factor. Nature. 273:718-723, 1978.

Schubert, D. and C. Whitlock, Alteration of cellular adhesion by nerve growth factor. Proc. Nat. Acad.

Sci. 74:4055-4058, 1977.

Slobota, R.D. and J.L. Rosenbaum, Decoration and stabilization of intact, smooth-walled microtubules with microtubule-associated proteins. Biochemistry. 18:48-55, 1979.

Smalheiser, N.R., S.M. Crain, and L.M. Reid, Laminin as a substrate for retinal neurons in vitro. Dev. Brain Res. 12:136-140, 1984.

Solomon, F. and M. Magendantz, Cytochalasin separates microtubule disassembly from loss of asymmetrical morphology. J. Cell Biol. 89:157-161, 1981.

Spero, D.A. and F.J. Roisen, Neuro-2a neuroblastoma cells form neurites in the presence of taxol and cytochalsin D. Dev. Brain Res. 23:155-159, 1985.

Theonen, H. and Y.A. Barde, Physiology of nerve growth factor. Physiol. Rev. 60(4):1284-1335, 1980.

Tischler, A.S. and L.A. Greene, Morphological and cytochemical properties of a clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Lab Invest. 39:77-79, 1978.

Tsui, H.C.T., K.L. Lankford, H. Ris and W.L. Klein, Novel organization of microtubules in cultured central nervous system neurons: formation of hairpin loops at ends of maturing neurites. J. Neurosci. 4(12):3002-3013, 1984.

Turner, D.C., L.A. Flier and S. Carbonetto, Magnesium-dependent attachment and neurite outgrowth by PC-12 cells on collagen and laminin substrata. Dev. Biol. 121:510-525, 1987.

Vlodavsky, I., A. Levi, I. Lax, Z. Fuks and J. Schlessinger, Induction of cell attachment and morphological differentiation in a pheochromocytoma cell line and embryonal sensory cells by extracellular matrix. Dev. Biol. 93:285-300, 1982.

Yamada, K.M., B.S. Spooner and N.K. Wessels, Axon growth: role of microfilaments and microtubules. Proc. Natl. Acad. Sci. 66:1206-1212, 1970.

Yamada, K.M., B.S. Spooner and N.K. Wessells, Ultrastructure and function of growth cones and axons of cultured nerve cells. J. Cell Biol. 49:614-635, 1971.

Yu, M.W., N.W. Tolson and G. Guroff, Increased phosphorylation of specific nuclear proteins in superior cervical ganglia and PC12 cells in response to nerve growth factor. J. Biol. Chem. 255:10481-10492, 1980.