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Tension as a Regulator of Neurite Outgrowth in Neurons from the Central Nervous System.

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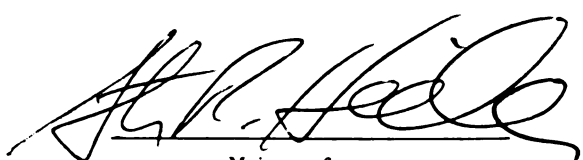
Master's degree in Physiology



Major professor

Date 8/21/96

TENSION AS A REGULATOR OF NEURITE OUTGROWTH IN NEURONS FROM THE CENTRAL NERVOUS SYSTEM

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Physiology

1996

ABSTRACT

TENSION AS A REGULATOR OF NEURITE OUTGROWTH IN NEURONS FROM THE CENTRAL NERVOUS SYSTEM

By

Sandeep R. Chada

This thesis investigates whether the development of brain neurons is closely tied to mechanical tension as previously shown for peripheral neurons. Nerve cells, obtained from cerebral hemispheres of 7-8 day-old chick embryos, were plated at low density. An *in vitro* method utilizing glass microelectrodes was used to quantify the direct relationship between applied tension and growth rate of neurites. Chick forebrain neurons are able to initiate neurites *de novo,* and elongate at a rate which is directly related to the amount of applied tension. The tension-induced neurites manifest a growth cone capable of independent motility and a dense axial array of microtubules. These neurons are very easy to culture, and extend a single long process of uniform caliber and several short tapering processes, each undergoing a stereotypic change in morphology. Therefore, this cell culture model may be especially useful in studying neuronal development *in vitro.*

dedicated to my mother and father

ACKNOWLEDGMENTS

I would like to thank Dr. Steve R. Heidemann for his support and guidance. Also, I would like to thank my colleagues Ching-Ju lin, Philip Lamoureux, and Dr. Jing Zheng for their assistance. I would like to especially thank my brother who provided encouragement and motivation throughout my graduate work.

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INTRODUCTION

Mechanical tension has been demonstrated to be a potent regulator of neurite outgrowth in chick sensory neurons and pheochromocytoma PC12 cells, which develop multiple axon-like process but do not establish axonal/dendritic polarity. The rate of axonal elongation in peripheral neurons was found to be a simple linear function of the applied force (Zheng *et al.* 1991; Lamoureux, *et al.*, 1992). Cultured dorsal root ganglion neurons from the chicken embryo have been used to show that tension is a regulator of four different phases of axonal development: 1) initiation 2) growth-cone mediated elongation 3) towed growth 4) axonal retraction (for review see Heidemann and Buxbaum, 1994).

For more than a hundred years, after Ramón y Cajal (1890) originally named the tip of the growing nerve fiber the growth cone, and from the time Ross Harrison (1910) invented tissue culture to study axonal growth, the growth cone has primarily been thought to be responsible for growth of nerve fibers. Paul Weiss (1941) categorized the growth of nerve fibers *in vivo* into three successive stages and suggested that tension might regulate axonal elongation. The first two stages "pioneering" and "application" are mediated by growth cone activity. During the first "pioneering" stage emerging fibers extend into the surroundings, and then in the second "application" stage subsequent fibers follow the path laid down by the pioneering fibers. The growth cone finds its target during the final "towing" stage following synapse formation the migrating target cells pull on the attached axon lengthening the neurite.

In culture, where the neurons can be observed continuously, it is generally agreed that motile, amoeboid activity is initially characteristic of

most, if not all, of the cell margin. Zheng *et al.* (1991), initiated neurites *de novo* by applying tension to the margins of chick sensory neurons using calibrated glass needles. Cultured sensory neurons were induced to elongate at 4-5X the fastest physiological rate, i.e. the fastest rate supported by growth cone advance, for several hours by towing with a needle at constant force. The experimentally initiated neurites contained a normal array of microtubules as observed by immunofluorescence and by electron microscopy (Zheng *et al.* 1993). Recently, Miller and Joshi, (1996) provided the critical evidence which supports the idea that neurite growth results from polymerization of tubulin monomers within the axoplasm not reorganization of existing microtubules. This suggests that mechanical tension applied to neurons directly stimulates microtubule assembly.

In a newly forming axon or dendrite, can the coupling between growth-cone locomotion and axonal elongation be simply that of mechanical tension? The cytoskeleton consisting mainly of filamentous actin, microtubules, and intermediate filaments provides the structural support for the cell. Given the arrangement of actin and microtubules in axons (Schnapp and Reese, 1982), a complementary force interaction between compressed axonal microtubules supporting tensile axonal actin may regulate microtubule assembly by a thermodynamic mechanism integrating microtubule assembly with growth-cone advance (Buxbaum and Heidemann, 1988; Dennerll, *et al.*, 1988). This thermodynamic model suggests that microtubule assembly occurs due to a shift of compression away from the microtubules to the substratum as the growth-cone advances (Heidemann and Buxbaum, 1990).

Neurites initiated *de novo* have a "normally" functioning growth cone at the distal end of the neurite, characterized by highly motile lamellipodium

and filopodia, which are continuously extending and retracting. The orderly arrangement of organelles and fibers in the neurite is disrupted at the growth cone. Neurofilaments and microtubules diverge and splay out into the broadened base of the growth cone. The functions of the growth cone include primary attachment of the cell to the substratum (Bray, 1973), axonal guidance (Bentley and O'Connor, 1994), and production of traction force generated primarily by contracting filopodia (Trinkaus, 1985; Heidemann, 1990). In tissue culture, the retraction of filopodia has been shown to exert a force (Nakai, 1960); the tension produced by these contractions may be at least one mechanism that underlies neurite elongation (Bray, 1979, Lamoureux *et al.*, 1989; Heidemann, *et al.*, 1990,1991; O'Connor *et al.*, 1990).

Additional evidence for a role of tension in neurite initiation comes from Smith's (1994 a, b) studies of the initial outgrowth of neurites from chick sympathetic neurons grown *in vitro*. The formation of a neurite involves the contact of the tip of the filopodium with another cell, a large bead, or a substrate coated with laminin, followed by the invasion of cytoplasm from the perinuclear region. The tension that develops in the filopodium seems to initiate the movement of cytoplasm, since dilation of the neurite begins only after straightening of the filopodia following contact (Smith, 1994b).

Lamoureux *et al.*, (1989) provided the first direct evidence that the growth cone is pulling forward. Quantitative evidence was obtained by utilizing calibrated glass needles attached to the cell bodies of elongating neurons. They observed that growth-cone advance was accompanied by increased tension in the axon shaft. Subsequently, Heidemann *et al.* (1990) provided extensive evidence that growth cone filopodia are contractile and can exert substantial pulling forces. Thus, under normal culture conditions, the

neurite appears to elongate from the tension stimulus provided by the growth cone.

By "towing" neurites using glass microelectrodes, Dennis Bray (1984) was the first one to establish a paradigm examining the role of externally-applied mechanical tension on axonal growth in cultured sensory neurons. The majority of "towed growth" during axonal development results from the expanding skeleton of vertebrates. Neurons from the peripheral nervous system (PNS) are exposed to large "towed" growths after target attachment; a human motor neuron innervating muscle may extend an axon which is a meter in length in the adult, but only 1cm in a 8-week-old embryo (Bray 1984). Axonal growth in the central nervous system is also growth cone-mediated, however these neurons are not exposed to the same magnitude of "towed" growth as the peripheral neurons.

Zheng *et al.* (1991) adapted the techniques used by Bray (1984) and extended his work on tensile regulation of axonal growth. Glass microneedles were attached to growth cones of chick sensory neurons and force was applied resulting in axonal elongation occurring over the course of seconds and minutes and continuing far-above physiological rates for many hours. Axonal elongation rates were found to be a linear function of applied tension. Therefore, the addition of neurite mass is connected in some direct way with the movement of the growth cone. One direct link between growth cone motility and axonal elongation is clearly the tension exerted by the pulling growth cone (Lamoureux *et al.*, 1989).

The role of tension in neurons from the central nervous system has not previously been investigated. The brain and other centrally derived neurons undergo complex morphogenetic movements and rearrangements that must involve mechanical forces. Tension, acting upon neuronal processes that are

anisotropically oriented and asymmetrically connected to distant targets, can account for specific anatomical characteristics such as the particular folding patterns of cerebral and cerebellar cortex (Van Essen, 1996). Tension-induced morphogenesis provides an efficient developmental mechanism for achieving compact wiring (minimization of interconnections between cortical components), an important characteristic that is demonstrable in many parts of the adult nervous system (Cherniak, 1994). For example, the brain of vertebrates, and of most invertebrates, makes more anterior than posterior sensor-motor connections. To minimize the total length of peripheral nerve fibers, the brain should be placed as far forward as possible, as is in fact the case (Cherniak, 1995). Costs of longer neural connections include not only the volume of highly active tissue to be grown and maintained, but also increased signal propagation delays.

Development of axonal/dendritic polarity in chick forebrain neurons:

Most vertebrate neurons consist of the cell body (soma) and two kinds of processes, usually one axon and multiple dendrites which differ profoundly in shape and function. The dendrite is specialized for receiving impulses, and the axon conducts action potentials generated at the initial segment of the axon (axon hillock). The neuron, therefore, shows a certain degree of structural and functional polarity. The dendrite appears as an extension of the soma, containing the same organelles found in it. Dendrites are several times shorter than axons and start out rather thick at the base and taper rapidly, while axons are thinner at the origin, but maintain a relatively constant caliber (Peters *et al.*, 1991; Pannese, 1994). The composition of axons differs in many ways, including the lack of rough endoplasmic reticulum, Golgi apparatus, and ribosomes free in the cytoplasm (except in the initial

segment), and an exceptionally high density of microtubules and intermediate filaments (Peters *et al.*, 1991).

Hippocampal neurons from the embryonic rat in dispersed cell culture are presently the cell type of choice to investigate neuronal polarity *in vitro* (Bartlett and Banker, 1984; Dotti and Banker, 1988). Several other culture models are available for studies of neuronal polarity, including cultures derived from sympathetic ganglia (Higgins *et al.*, 1991), mesencephalon (Lafont *et al.*, 1992), and cerebellum (Caceres and Kosik, 1990). However, the neuronal populations in these cultures have not been fully characterized, and the extent to which they develop a mature, fully polarized phenotype remains uncertain. Given the diversity of neuronal forms and functions, the development of additional cell culture models expressing neuronal polarity will aid in elucidating the complex mechanisms involved in neuronal differentiation.

Dotti and Banker (1988) followed the early development of axons and dendrites in individual hippocampal neurons in culture by sequential photography and time-lapse video recording. They suggest that axonal polarity occurs in four distinct stages of development. The first few hours following plating the neurons attach to the substratum, and are surrounded by flattened lamellipodia (Stage 1). After several hours the lamellipodia condense to form several discrete minor processes, and cannot be distinguished either as axons or dendrites (Stage 2). About 12 - 24 hours after plating, one of the minor processes begins to grow at a rapid rate and establishes itself as the axon while the other processes become dendrites (Stage 3). The growth of dendrites in hippocampal neurons is much slower than that of the axon, and does not begin until about 4 days in culture (Stage 4).

Until now, all of the experiments studying the role of tension in axonal development have been performed on neurons from the peripheral nervous system or on differentiated PC12 cells. These studies suggest that neurite initiation and elongation is regulated by applied mechanical tension (Zheng, *et al.*, 1991, Bray, 1979). In view of this intimate relationship between mechanical force and axonal development, the purpose of this study is two fold: One, is to investigate the mechanical growth properties of central neurons in response to applied tensions; two, to determine if chick forebrain neurons undergo neuronal differentiation *in vitro*.

MATERIALS AND METHODS

Cell culture:

Cerebral hemispheres from seven or eight day-old chick embryos are dissected, cleaned of their meningeal membranes and dissociated by treatment with trypsin (0.05%) for a period of 15 min as described by Sensenbrenner *et al.*, (1978). The tissue is then rinsed three times (5 min each) with balanced saline solution (BSS) containing 10% fetal calf serum (v/v; Gibco Labs, NY). A fire-polished Pasteur pipette is used to triturate the cells until tissue is dissociated. To obtain a low density (1,500 - 2,000 cells/cm^2) of neurons, a desired amount of cell suspension ~0.2 ml and ~0.8 ml is placed into 35 mm and 60 mm plastic culture dishes, respectively. The dishes are prepared in advance by soaking them with poly-L-lysine (~1 ml) for 2 *hours* and then rinse three times (5 min each) with sterile water.

A low plating density was desired for two reasons: One, to avoid aggregates of cells, making it possible to view the complete form of a neuron; Two, to allow external manipulation of individual processes. The cells are plated in L-15 medium supplemented with 0.6% glucose, 2 mM glutamine, 100U/ml penicillin, and 100mg/ml streptomycin, and 20% FCS. The use of L-15 medium (Gibco Labs, NY) which uses hydroxyethyl piperazine ethane sulfonic acid (HEPES) as a buffer, instead of sodium bicarbonate to maintain pH at physiological levels (~7.4), permits cell growth without the addition of CO_2 to the ambient environment. 7S Nerve growth factor (100ng/ml; Harlan, IN) and N-2 supplements (10 µl/ml; Gibco Labs, NY) are added to the plating medium, and are incubated at 37° C.

Direct axial force measurement:

Glass needles are manufactured and calibrated for force as originally described by Yoneda (1960), with the following modifications. Two relatively stiff wire needles were made from 8- and 16- mm lengths of uniform diameter (25 μm) chromel wire (Omega Engineering, Inc., Stamford, CT). Each of the wires was calibrated for force by hanging weights of 0.5 to 2 mg from their tips to obtain the "calibration constant" for each of the wires. A third 24- mm wire needle could then be calibrated indirectly by using the following standard relation for beam deflection:

$$\text{Force} = \frac{(\text{constant})\ (\text{tip displacement})}{(\text{needle length})^3}$$

Using the values obtained for the constant from the two shorter (stiffer) wire needles a calculated value of 90.9 μdynes/μm was obtained for the third wire needle (Dennerll *et al.*, 1988).

Glass microneedles were fabricated from glass tubing (Drummond scientific; outer diameter 0.90 mm) placed in a BB - CH horizontal microelectrode puller (Mecanex, Geneva, Switzerland). The more flexible intermediate glass needles are then calibrated by bending them against the wire needle, and subsequently glass needles against each other, to obtain "pulling" needles with stiffness calibration constants of (3 - 10 μdynes/μm). The calibrated glass needles are pretreated by immersion in poly-L-lysine (1 mg/ml) in PBS, then in Concanavalin A (50 mg/ml) a plant lectin which has previously been used to study neuronal plasticity (Smith and Jiang, 1994); each immersion is for 30 min at room temperature. The needle is mounted in a single micromanipulator, capable of producing incremental changes in distance in the X, Y, and Z planes, with its tip a short distance from a second

reference needle. This second needle serves as a reference for measuring the deflection of the "pulling" needle, and possible drift of the micromanipulator system. The response of the cells to applied tension are recorded by time-lapse on videotape and subsequently analyzed for the level of applied tension and neurite growth. To maintain cells at physiological temperatures and to minimize thermal changes in micromanipulator position, the entire space surrounding the microscope is maintained at 37° C using an air curtain incubator.

Neurite Initiation and elongation:

When plated on poly-L-lysine (1 mg/ml) chick forebrain neurons attach to the substratum rapidly, and within 6-9 hours the cell bodies are surrounded at the periphery with lamellae. Cells at this stage are attached to glass microneedles (< 1 μm at the tip) and external tension is applied. The magnitude of tension is regulated by controlling the displacement of the force-calibrated "pulling" needle.

Neurites were elongated by attaching a calibrated needle to the growth cone of a minor process (present in neurons that have been in culture for 1 day). The neurite was then pulled in "steps" of constant force; that is, a tension magnitude was chosen beginning at 20 - 40 μdyn, and this tension was held constant for 20 - 50 min by moving the micromanipulator to maintain the appropriate deflection of the calibrated needle. Subsequently, the same technique was used to apply 20 - 50 min periods of higher tension to the neurite, each level typically 20 - 30 μdyn higher than the previous value. Some of the experimentally initiated and elongated neurites were examined by staining with anti-β-tubulin antibodies to detect the presence of microtubules.

Indirect Immunofluorescence:

Following a period of growth the initiated neurites are allowed to reattach to the dish, allowing the needle to be detached from the growth cone. The neuron is then marked by circling the bottom of the culture dish with a diamond-tipped "objective". Microtubules are "stained" for immunofluorescence using a method similar to that of Thompson *et al.*, (1984). The cell is first permeabilized with 0.5% Triton X-100 (v/v) in microtubule-stabilizing buffer (MTSB) for 3 min at 37°C. The detergent lyses the cell membrane and allows antibodies to enter the cytoplasm. The Triton X-100 is then removed from the dish and 10 ml of 3.7% formaldehyde (v/v) in MTSB is added for 10 min at 37°C. Alcohol dehydration, with 100% methanol for 10 min at 20° C, is performed following the fixation. The cells are then rehydrated in phosphate buffered saline (PBS) for 1 min at room temperature. The primary antibody (mouse anti-beta tubulin; Amersham, Arlington Heights, IL) is diluted to a concentration of 1/500 in PBS containing bovine serum albumin (BSA; 0.5%) and sodium azide (0.1%). A drop of the primary antibody is placed on the cell, and the dish is stored overnight at room temperature in a moist chamber. The next day the sample is rinsed, and is then incubated with fluorescently-labeled secondary antibody (goat anti-mouse IgG; KPL Laboratories Inc., Gaithersburg, MD) diluted to a concentration of 1/20 in PBS containing BSA (0.5%) and sodium azide (0.1%), at 37° C for four hours. The cells are observed through an Odyssey confocal microscope (Noran Instruments, Middleton, WI) equipped with fluorescent optics.

Light microscopic study of chick forebrain neurons in culture:

The neurons are plated at low density, on poly-L-lysine (0.1%) coated

culture dishes, to study isolated cells. The cells were selected and circled with a diamond objective marker so that they could be relocated, and the processes from the selected cells are followed by visual examination. Observations of individual cells are made at various stages of cell growth by phase-contrast microscopy and recorded on video tape. Since the cells are plated at low density, the complete form of the neuron is visible without the need for any special marking techniques.

Transmission electron microscopy:

Ultrastructural analysis of chick forebrain neurons in culture is performed at different stages of growth. The cells are fixed with 2.5% gluteraldehyde in L-15 media for 30 min at 37° C. Postfixation is performed for 5 min with 1% osmium tetroxide in 0.1 M cacodylate buffer. The cells are rinsed with cacodylate buffer, followed by three rinses with distilled water. Cells are stained with 2% aqueous uranyl acetate for 2 hours. Uranyl acetate intensely stains nucleic acids and proteins (Pannese, 1994). The cells are dehydrated in a graded series of ethanols and then embedded in Polybed 812 (Polysciences, Inc., Warrington, PA) resin. Polymerization of the resin is facilitated by incubating the embedded cells for 1 day at 40°C and then for an additional day at 60°C.

Following polymerization, the resin is removed from the culture dish leaving the neurons at the bottom surface of the resin block. Cells which resemble a polarized neuron with one long process and several shorter processes are selected, circled with a diamond object marker, and cut out using a fine saw. The block containing the desired cell is then trimmed for thin sectioning. Thin sections ($\leq$ 80 nm) are cut parallel to the substrate using the LKB ultramicrotome (LKB Produkter AB, Stockholm, Sweden) with a

diamond knife, and collected on 150 mesh copper grids. The cut sections are post-stained with lead citrate (LC; 0.05%; w/v) for 3 min in a chamber containing 0.1 % sodium hydroxide to remove CO_2 from the immediate environment Lead citrate can form lead carbonate crystals on the sections if it is allowed to react with CO_2 (Racker, 1983). The stained sections are then viewed under the Phillips CM-10 transmission electron microscope (at a voltage of 60 kV).

RESULTS

Central nervous system neurons in culture were able to initiate neurites and elongated in response to mechanically-applied tension. *De novo* initiation of neurites from cultured chick forebrain neurons was induced by applying external tension tangent to the margin of the cells in random locations (Figure 1). Glass microneedles were pre-treated with adhesive proteins to promote cell adhesion. The initial photomicrograph depicts the "pulling" needle being attached to the cell body (Figure 1a, the reference needle is slightly out of focus). The distance between the reference and "pulling" needle provides a direct measure of axial force applied to the cell body. Tension was applied very gradually and after an hour a uniform caliber cytoplasmic process formed proximal to the pulling needle. Growth of the neurite may occur due to cortical flow of cytoskeletal elements from the cell body or maybe by polymerization of cytoskeletal elements in the axoplasm (Figure 1b). After 3 hours, the neurite reached a final length of ~75 μm and the needle was detached from the growth cone (Figure 1c). Immunocytochemistry using fluorescently labeled antibodies against β-tubulin, demonstrated the presence of intact microtubule arrays within the initiated neurite (Figure 1d). The intensity of staining within the experimentally initiated neurite is similar to that of a control neurite.

Neurite initiation occurred either at the site of needle attachment or opposite the site of attachment. Figure 2 displays the sequence of events leading to the initiation of a neurite distal to the cell body. Initially the "pulling" needle is manipulated on to the cell; following attachment to the cell body tension is applied, resulting in the movement of the soma and growth of a neurite distal to the applied tension (Figure 2b). The tension required to initiate

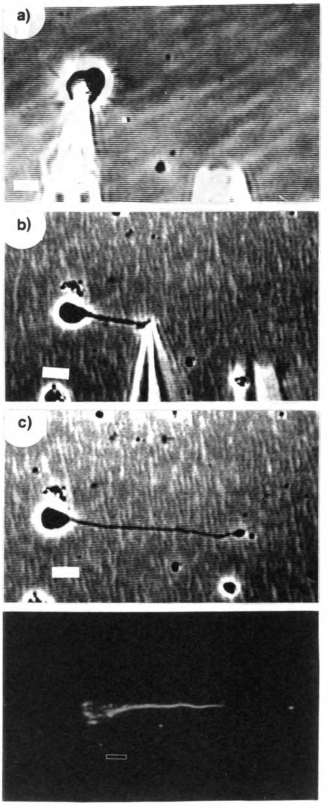


Figure 1: *De novo* initiation from chick forebrain neurons by external application of mechanical tension. These video micrographs were taken in sequence at different time intervals. The experiment began ~8 hours after plating the cells. The first micrograph (A), depicts the start of the experiment; the pulling needle, attached to the soma and the reference needle (slightly out of focus) are displayed. (B), After 40 min, a neurite of uniform caliber is initiated proximally and a growth cone seems to form at the site of needle attachment. (C), The neurite grows to a final length of ~ 75 μm. The calibrated needle is then detached and the cell is fixed and processed for immunofluorescence. (D), A confocal image of the cell following immunofluorescent staining using antibodies against β–tubulin demonstrates the presence of microtubule arrays throughout the length of the initiated neurite. (Scale bar = 10 μm)

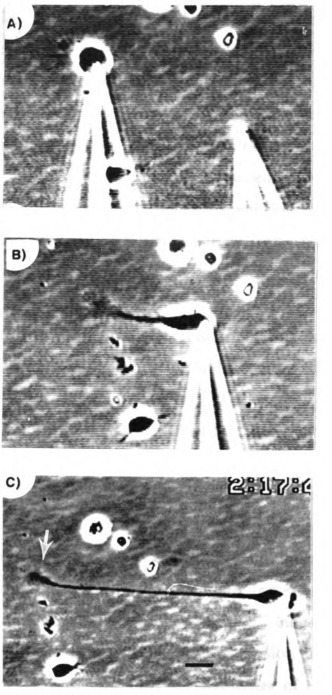


Figure 2: Sequential phase contrast photomicrographs of initiation of a neurite distal to the cell body from a chick forebrain neuron in culture. The experiment began 8 hours after plating the cells. (A) Depicts the start of the experiment; the "pulling" needle (attached to the cell body) and reference needle (slightly out of focus) are displayed. (B) After approximately an hour the cell body is firmly attached to the needle and a neurite of uniform caliber begins to initiate distal to the cell body. (C) After three hours the length of the neurite reached ~90 μm, and a functional growth cone can be observed (arrow). (Scale bar = 10 μm)

this neurite is ~180 μdynes; this is within the range of tension exerted on the cell body by the pulling growth cone (Lamoureux *et al.*, 1989). As the neurite is initiated, a functional growth cone (arrow) develops at the distal tip (Figure 2c). Microspikes are observed to protrude and retract appearing to "search" the substratum. A neurite with a final length of ~90 μm is initiated in a total of 2 hours. The tension required to initiate neurites distal to the cell body is much greater than that required to initiate neurites on the proximal side as is illustrated in Table 1. The tension required to initiate neurites from chick forebrain neurons is 4 - 6 times lower than that of chick sensory neurons (Zheng, *et al.*, 1991).

The quantitative relationship between elongation rate and applied tension for chick forebrain neurons was determined by using a method similar to that described by Zheng, *et al.*, (1991) for chick sensory neurons. The neurites of chick forebrain neurons (in culture for 1 day) were attached by their growth cones to calibrated glass needles and were subjected to increasing tensions as steps of constant force. As shown in Figure 3, each step lasted 20 - 50 min. and was 20 - 40 μdynes greater than the previous step (Figure 3, open circles). Rather than equilibrating to a given force, the neurites of forebrain neurons elongated continuously in response to applied tensions with increasing elongation rate at increased tension levels (Figure 3, filled circles). Indeed, the neurites show a simple linear relationship between applied tension and elongation rate, with the slope of the line represents the sensitivity of the neurite (Figure 4a and 4b). Immunocytochemistry using antibodies against ß-tubulin suggests that such "towed" neurites contained normal, high density microtubule arrays, entirely similar to spontaneous neurites (as illustrated in Figure 5).

Table 1: List of applied tensions required to initiate neurites from chick forebrain neurons in culture:

Initiation of Neurite Proximal to the Pulling Needle (N = 14)	Initiation of Neurite Distal to the Pulling Needle (N = 14)
73 μdynes	308 μdynes
10	326
81	50
33	223
5	75
18	93
54	196
37	291
16	28
32	58
16	125
18	107
25	25
18	69

Mean $\pm$ S.D. = 31 $\pm$ 23 μdynes Mean $\pm$ S.D. = 127 $\pm$ 96 μdynes

Figure 3: Neurite lengthening in response to increasing steps of tension. Extant neurites from chick forebrain neurons were "towed" by their distal ends by a calibrated glass needle. The filled circles represent the overall neurite length (left ordinate), in response to the applied force (right ordinate).

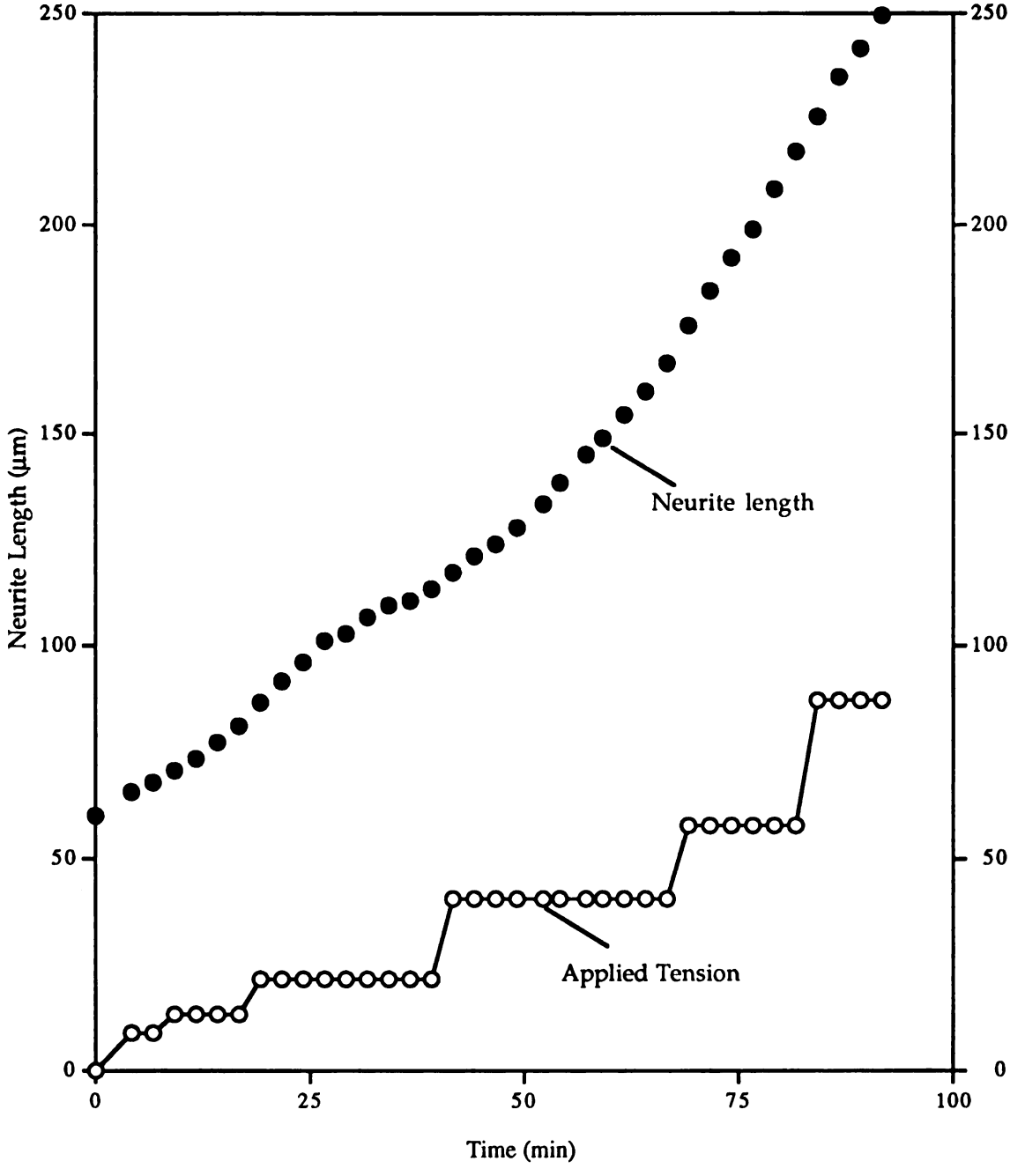
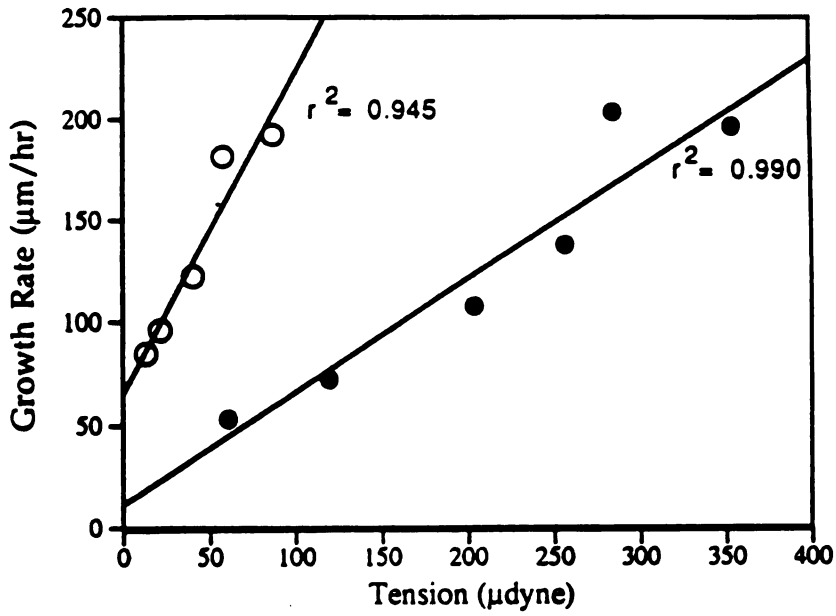
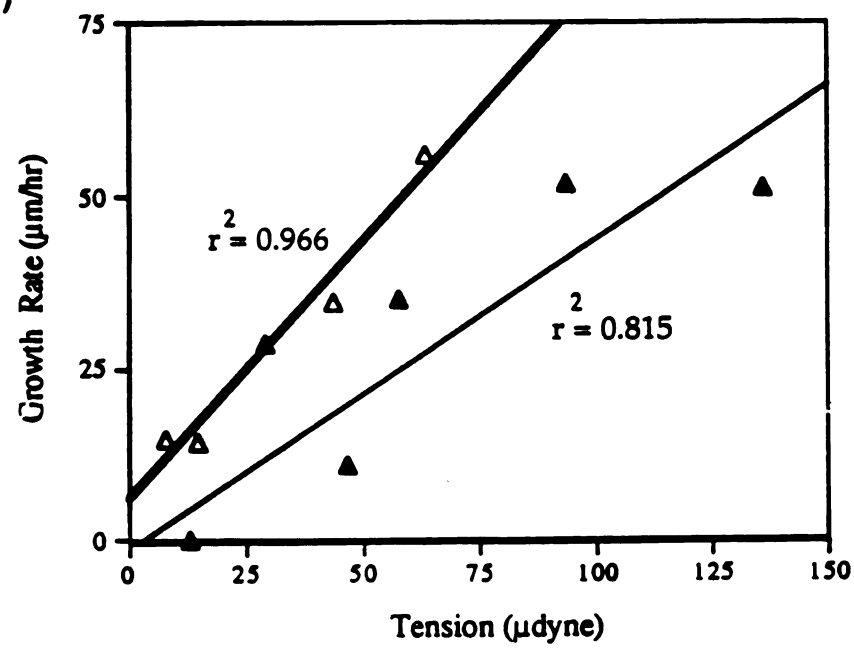


Figure 4: Neurite growth rates as a function of experimentally applied tension for both the initiated (Fig. 4a) and "towed" neurites (Fig. 4b). Each line reflects the data from a single experiment. The slopes of these lines are the sensitivities of the neurites to tension (their growth rate per μdyne of tension). The coefficient of correlation for each of the lines is displayed within each of the figures.

a)



b)



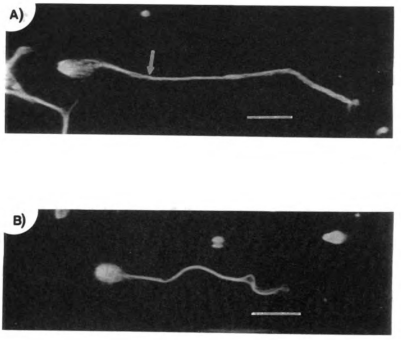


Figure 5: Immunofluorescent images from the confocal microscope using antibodies against ß-tubulin on a neurite "towed" experimentally (A), or on a neurite which emerged spontaneously (B). The arrow in (A), indicates the site where "towing" was initiated. The initial length of the extant neurite was ~11 μm (the arrow indicate site of needle attachment), and the final length following "towed" growth was ~ 95 μm. (Scale bar = 10 μm)

The development of axo/dendritic polarity based on gross morphology:

Chick forebrain neurons in cell culture for 4-5 days develop the morphology characteristic of axonal/dendritic polarity of the corresponding neuronal cell type *in vivo* . Figure 6 is a phase-contrast photomicrograph of a forebrain neuron which has been in culture for 5 days. Morphologically this neuron appears to have differentiated two types of processes, with one long uniform caliber process and several short tapering processes. This is a well described characteristic of axons and dendrites in polarized neurons (Peters, *et al.* 1991; Pannese, 1994). This polarized morphology develops over a time course very similar to that described for hippocampal neurons (Dotti and Banker, 1988), the model system for studying the cell biology of axonal/dendritic polarity.

Examples of chick forebrain neurons in different stages of growth (as classified by Dotti and Banker, 1988) are presented in sequential photomicrographs (Figure 7). In the early stages of growth (stage 1) the cells are rounded and are surrounded by *lamellae* at the periphery (cell marked by curved arrow in Figure 7a; 1 day in culture). The two cells marked with arrowheads are in stage 2 of growth, and extend several small (minor) processes which are similar in appearance (Figure 7a). These minor processes are very active and are continually advancing and retracting. One of the minor processes then enters a period of rapid growth to form an axon-like process (Stage 3). The arrow in Figure 7a points to the long axon-like process emanating from a cell (marked by a star) in stage two/three. This axon-like process (arrows) has grown ~180 μm in just 1 day in culture, while the other minor processes are less than 40 μm in length. At 2.5 days in culture the axon-like process continues to grow rapidly (~500 μm) as do some of the minor processes (Figure 7b). In cells that have been observed at this stage

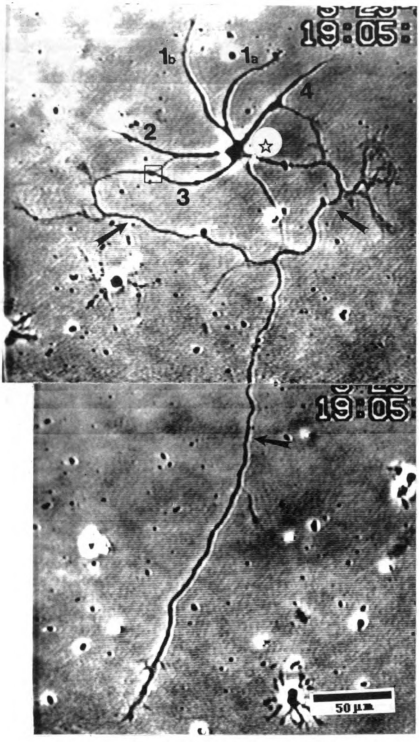


Figure 6: Photomicrograph of a differentiated neuron 5d in culture. Arrows point to the branched axon-like process (~500 µm in length). The dendrite-like processes are numbered from 1 - 4. (Scale bar = 50 µm)

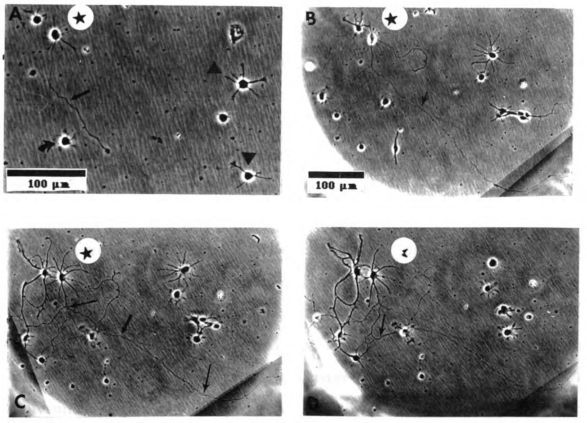


Figure 7: Stereotyped growth of chick forebrain neurons in culture. A series of phase-contrast photomicrographs taken in sequence (at specified intervals), illustrates the possible establishment of axonal/dendritic polarity. Several neurons are displayed in (A) (~1d in culture), some neurons seem to develop at a faster rate than others. The cell marked by a curved arrow is in stage 1 of development (is surrounded by lamellae). The cells marked by arrowheads have developed several short (minor) processes which are similar in appearance. The cell marked with the star has undergone rapid growth, one of the minor processes has elongated tremendously and in appearance resembles an axon (arrow). (B), After 2.5 days in culture the major process ("axon") of the neuron, marked by a star, continues to grow rapidly. The other minor process, however, remain largely stationary. At 4.5 days in culture (C), the major process (demarcated by arrows) begins to branch and send collateral branches to adjacent cells. (D), After 6 days in culture, the demarcated cell begins to have extensive contact with the adjoining cell and the "dendrites" appear slightly enlarged.

only one long process is present, and a second long process has not been observed to develop. The majority of "dendritic" growth (stage 4) occurs between 2.5 days and 4.5 days (Figure 7c). Also, the axon-like process begins to branch and contact adjacent cells, and seems to be benefiting from these interactions. At this stage the cell begins to resemble a polarized neuron with axons and dendrites. At 6 days in culture (Figure 7d) the axon is extensively branched, and the dendrite-like processes remain the same length.

Electron microscopic study of ribosomal density provides support for the establishment of polarity in chick forebrain neurons:

Neurons in the maturation stage (day 5-6 in culture) which display the gross morphological characteristics of a polarized neuron (*i.e.* one long process and several short processes), were examined for the presence of polysomes in dendrite-like processes. Preliminary results suggest that the number of polysomes is greater in the dendrite-like processes. The contrasting distribution of organelles and cytoskeletal elements in the presumptive dendrite and axon are illustrated by representative micrographs. The distal portion of dendrite #2 (~60 μm from the soma) is illustrated in Figure 8a, clusters of polysomes associated with vesicular elements and free in the axoplasm are visible. A branch of the presumptive axon (at ~250 μm from the soma) shows the absence of polysomes, while the neurofilaments which run longitudinally through the axon can be easily viewed in Figure 8b.

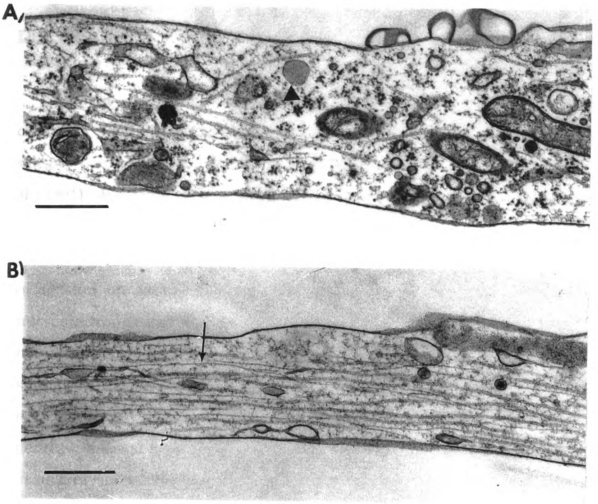


Figure 8: Representative micrographs of contrasting dendrite-like and axon-like processes from a chick forebrain neuron in culture for 5 days (see Fig. 1). (A), Is the distal region of a dendrite-like process, clusters of ribosomes are indicated (arrowhead), and rough endoplasmic reticulum appears to be present and is seen as membranous vacuoles. (B), Is a segment of the axon-like process from the same neuron (~250 μm). Polysomes are absent and neurofilament arrays are abundant (indicated by an arrow). (Scale bar = 0.5 μm)

DISCUSSION

Initiation and elongation of neurites from chick forebrain neurons in culture is closely linked to applied tension, as previously described for chick sensory neurons and PC12 cells (Bray, 1984; Dennerell *et al.*, 1989; Zheng, *et al.*, 1991). We found that the magnitude of tension is directly related to the elongation rate for both the initiated and "towed" neurites. This simple, fluid-like relationship between elongation and tension suggests a direct effect of tension on axonal assembly. Externally-applied tension using glass microelectrodes can initiate neurites *de novo,* simply, by pulling on the margins of chick forebrain neurons in culture. The tension-induced neurites, similar to neurites initiated from neurons from the peripheral nervous system (Zheng, 1991), manifest two important characteristics of normal neurites; a distal growth cone capable of independent motility and a dense axial array of microtubules throughout their length similar to spontaneous neurites from these cells (Figure 1d; Figure 5). Formation of growth cones typically occurs rapidly at the site of needle attachment or at the terminal end of a neurite initiated distally.

About half the time, as tension is applied, the cell margin remains fixed and the soma moves forming a neurite opposite the site of attachment. As illustrated in Table 1, the tension required to initiate neurites distal to the cell body is much greater (~170 μdynes) than the tension required to initiate a neurite proximal (~30 μdynes) to the calibrated needle. Chick forebrain neurons in culture are a relatively homogenous group of cells, except the maturation rate of the cells varies i.e., some neurons emanate processes earlier than others. Initiation experiments are performed at a very early stage (6 - 8 hours after plating) before process outgrowth. Cells that initiate a

neurite proximal to the pulling needle may be at a slightly more advanced stage in development, therefore requiring less time and tension to initiate a neurite. On the other hand, distal initiation may be a result of mechanically "forced" induction of a neurite from a less developed neuron, therefore requiring more time and tension to initiate process outgrowth.

Forebrain neurons in culture for one day posses minor processes extending from the cell body. Application of tension, using a calibrated glass needle, to the growth cone of a minor process is an *in vitro* analog of "towed" growth *in vivo*, initially described by Weiss (1941). The elongation rate of the neurites is proportional to the applied tension; the process elongates continuously in response to mechanical applied tension as increasing steps of constant force (Figure 3, filled circles). Plotting neurite growth rate as a function of applied tension results in a nearly linear plot, with the slopes of the lines representing the sensitivity of the neurites to tension (Figure 4).

Neurite growth in central neurons in response to force seems to resemble a fluid response over short time scales, with neurite advance being directly proportional to tension. Forebrain neurons are more sensitive to tension than sensory neurons, they achieve similar elongation rates at 4 - 6X lower tensions. The enhanced response of chick forebrain neurons to tension may be due to the fact that central neurons *in vivo* are subjected to much lower forces than peripheral neurons. Initiation of neurites from central neurons provides additional evidence for the idea that mechanical force can both initiate neurites and regulate microtubule assembly. Tension generated by the growth cone may activate signal transduction pathways which could then regulate cytoskeletal assembly and axonal/dendritic growth. However, the mechanism by which mechanical force informs and affects chemical reactions of growth is not well understood.

Chick forebrain neurons as a model system to study the tension-based theory of morphogenesis:

The ease and reliability of culturing chick forebrain neurons make this model ideal for investigating the role of tension in regulating axonal growth *in vitro*. Tension has previously been suggested to play a role in the morphogenesis of the peripheral nervous system (Bray, 1979). Potential role of tension in the central nervous system has received surprisingly little consideration. In gyrencephalic species, the increase in cortical surface area outpaces the growth of the underlying subcortical volume, requiring the cortex to become convoluted. Cortical folding could occur when large numbers of axons connected to nearby regions pull in a common direction, leading to bulk tissue displacement maintaining proximity between regions which are strongly interconnected (Van Essen, 1996). This tension-induced folding should tend to keep the neuronal circuitry compact even as the brain expands greatly in size. It has been hypothesized that specific areas of the cerebral cortex are placed to minimize the total length of their interconnections (Cherniack, 1995).

Another role for tension induced morphogenesis might be during the earliest events of neurogenesis. A fundamental property of the developing central nervous system is that neurons migrate from their sites of origin in germinal centers to more or less distant final positions (Sidman and Rakic, 1973; Jacobson, 1991). For example, neurons destined for the neocortex are generated in the ventricular and subventricular zones of the forebrain. On completing their final mitoses, most of the cortical neurons migrate substantial distances across complex terrains in order to reach their destinations within the cortex (Caviness and Rakic, 1978). Each neuron will

reside in a certain cortical layer, defined by the characteristic size and morphology (McConnell, 1988). Postmitotic neurons from the ventricular zone migrate up the processes of radial glial fibers, maintaining an intimate apposition to these fibers in the proliferative zone until they reach the cortical plate (Rakic, 1972). The mechanism by which young neurons "scale" the glial fiber is unknown. Differential adhesive affinities of the cells may be responsible for the segregation patterns of different neuronal classes of cortical neurons. Such a mechanism might be similar in principle to that thought to govern the segregation of heterogeneous cell populations in tissue culture (Caviness, 1977).

Possible establishment of neuronal polarity in chick forebrain neurons:

An additional aspect of the reported work is the potential use of forebrain neurons for the study of axonal/dendritic polarity. Neuronal polarity is reflected in the difference between the morphology of axons and dendrites, in the organization of the cytoskeleton and membrane specialization's, and in the absence of protein synthesis in the axon. Hippocampal neurons from the embryonic rat are currently the standard cell type used to study axonal/dendritic polarity *in vitro*. However, the hippocampal neurons are quite difficult to culture, in that they need to be cocultured with glial cells, and this requires much preparation time and expense. Forebrain neurons on the other hand can be dissected and cultured within a hour, and do not require the presence of glial cells for growth. We find that chick forebrain neurons extend two discrete processes, a single long process of uniform caliber and several short tapering processes, by day 5 in culture (Figure 6). Each of the processes undergoes a stereotypic change in morphology during *in vitro* development Experimentation for extended periods is required when

using glass microelectrodes for micromanipulation, therefore chick forebrain neurons which grow in the absence of carbon dioxide are more suitable for our experimental paradigm.

Preliminary observations of chick forebrain neurons in culture suggests that the growth and differentiation of these neurons is very similar to that of hippocampal neurons *in vitro*. Initially, both cell types establish several short, apparently identical minor processes; of these, only one acquires "axonal" characteristics, the remainder become "dendrites" (Figure 7). The fact that only one of the minor processes begins to grow rapidly and extends into a process which is many times longer than the others, and is of uniform caliber, suggests that the neuron may be undergoing neuronal differentiation. The viability of neurons in culture is dependent on the plating density (Banker and Goslin, 1991), therefore in these low density cultures some cell death is apparent, possibly due to a lack of contact-mediated interactions that occur during the cells' normal development *in situ* but may not occur *in vitro* It is apparent from these morphological observations that chick forebrain neurons seem to posses axonal/dendritic polarity. However, we are still not certain if the polarized appearance of these neurons reflects the cytoplasmic specialization present in axons and dendrites *in situ*.

Cytoplasmic specialization with respect to protein synthesis also seems to exist within the two processes. Axon-like processes contain few ribosomes compared to the dendrite-like processes, when examined under the electron microscope (Figure 8). It is difficult to judge the consistency of this difference from individual examples such as these. However, a large fraction of this cell could be studied electron microscopically, therefore, it is possible to determine the density of polysomes throughout much of the cells' arborization The asymmetric distribution of ribosomes within the two types of processes

provides preliminary evidence for the differentiation of chick forebrain neurons into axons and dendrites; other differences exist between axons and dendrites and these also need to be investigated before this cell type can be said to exhibit neuronal differentiation.

Future questions to be addressed to study the establishment of neuronal polarity:

Many structural and functional differences have been identified between axons and dendrites, however no ultrastructural feature that predicts whether a minor process would become an axon has been identified. The following questions on the genesis of neuronal polarity still perplex us: What is the mechanism by which axons are differentiated from dendrites? How do the various components within neurons develop to ensure polarity? To what extent is polarity intrinsic and to what extent and by what means is polarity determined by external conditions?

Conclusion:

Based on previous *in vitro* studies on sensory neurons (Heidemann, 1996; Zheng, *et al.*, 1991; Bray, 1984), and the current investigation of forebrain neurons, mechanical tension is likely to play an important role in the cellular development of process outgrowth, as well as in the interactions among neurons underlying brain morphogenesis. Preliminary results clearly indicate that the qualitative relationships between tension and process outgrowth are similar in chick sensory and forebrain neurons. The forebrain neurons are also very easy to culture and do not require glial cells for growth. Therefore, chick forebrain neurons could potentially be a valuable resource for investigating mechanisms underlying axonal/dendritic development *in*

vitro, and could also provide a suitable model for the study of the biochemical and functional maturation of neuronal cells in a well defined molecular environment.

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