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EFFECTS OF CELL ADHESION MOLECULES AND EXTRACELLULAR MATRIX MOLECULES ON GROWTH CONE MOTILITY AND PATHFINDING

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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January, 1995
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GRADUATE STUDIES

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candidate for the Doctorate

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(signed) Mrs. Pentikainen
(chair)

[Signatures]

date 7/30/97

*We also certify that written approval has been obtained for any proprietary material contained therein.
EFFECTS OF CELL ADHESION MOLECULES AND EXTRACELLULAR MATRIX MOLECULES ON GROWTH CONE MOTILITY AND PATHFINDING

Abstract

by

SUSAN BURDEN GULLEY

During development, neurons establish synaptic connections in a precise and specific manner. In many cases, axons must traverse long distances through a complex environment comprised of a variety of cell types before reaching their target tissues. During this outgrowth period, the terminal region of the axon possesses a membranous structure called a growth cone. The growth cone is highly motile, and acts as a sensory organ that interacts with other cells and molecules in its environment. A variety of cell adhesion molecules (CAMs) and extracellular matrix (ECM) molecules are encountered during this outgrowth period. Growth cones express receptors for many of these molecules on their surface, and thus may receive instructive influences from contact with certain CAMs and ECM molecules during growth toward their target tissues.

In order to gain an understanding of how CAMs and ECM molecules influence growth cone pathfinding, several \textit{in vitro} experiments were performed. The first group of experiments examined the ability of several substrate-bound CAMs and ECM to affect multiple growth parameters such as growth cone adhesivity and choices neurites make when presented with two substrates. The results of these experiments showed
that substrate adhesivity did not correlate with growth rates, degree of neurite
fasciculation, or substrate preference. Therefore, substrate adhesivity is not a principal
factor in axonal pathfinding.

A second group of experiments utilized timelapse videomicroscopy to examine
behavioral and morphological changes in growth cones upon encountering sharp
borders between substrates. The results of these experiments showed that growth cone
morphology is actively affected by the substrate. In addition, a large percentage of
growth cones collapsed upon contact with a new substrate, indicating that collapse may
be a normal response to molecules in the growth environment that are of a non-
inhibitory nature. These results suggested that growth cone interactions with CAMs
and ECM molecules may result in the generation of distinct intracellular signals that
mediate different behavioral responses of the growth cone.

A third set of experiments examined the distribution of cytoskeletal elements in
growth cones growing on different CAM or ECM substrates. These experiments
showed that cytoskeletal elements are distributed in unique patterns dependent upon the
growth substrate. In growth cones interacting with a border between two substrates,
the cytoskeletal elements underwent a progressive transition to a distribution pattern
appropriate for the new substrate. These results suggest that CAMs and ECM
molecules may have direct effects on the cytoskeleton of growth cones that underlie the
morphological changes observed with timelapse videomicroscopy.

Together, the results described here provide new information about how growth
cone motility is affected by CAMs and ECM molecules. In addition, these results
suggest an interaction between CAM and ECM receptors and the underlying
cytoskeleton. Therefore, although CAMs and ECM molecules function as permissive
highways for axonal growth, they may also provide instructive information that is
interpreted by individual growth cones and utilized for directed growth in vivo.
DEDICATION

I would like to dedicate this thesis to two separate groups of people:

First, to my parents, who provided immeasurable support, encouragement and love. They helped me to develop self-confidence that was key in my willingness to attempt many challenges.

Second, to my grandmother, Mrs. Grace Burden, and my mother-in-law, Mrs. Margaret Gulley. They have taught me a great deal about what is important in life, and have demonstrated that integrity and compassion can go hand-in-hand with success.
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First, I would like to thank my thesis adviser, Dr. Vance Lemmon. Dr. Lemmon has an amazing ability to exude excitement about science, and this has had a powerful effect on me. From the first time I met him, I knew I would like to work with him. As I have gotten to know him, I have realized that he represents the honesty and integrity that I had hoped to find in science. He has promoted the type of sharing lab environment I would like to someday mimic, and has been very supportive of his lab members in multiple ways. Most importantly, he refrained from using red pen when editing my written work.

I would also like to thank the members of my thesis committee, Dr. Lynn Landmesser, Dr. Gary Landreth, Dr. Bob Miller and Dr. Urs Rutishauser, for their helpful suggestions and support of my project.

The members of the Lemmon lab helped to make the last four years truly enjoyable. Dr. Ross Payne was instrumental in my learning how to use the image analysis system. His endless patience and willingness to demonstrate a variety of techniques were greatly appreciated. Eric Wong and Andy Schaefer are wonderful friends who helped me stay sane when the experiments were not cooperative. I spent hours discussing science and life-related issues with them, and always acquired a new perspective. Wendy Elmslie and Guang-hui Cheng not only provided a great deal of help preparing reagents, but friendship as well. I feel lucky to have interacted with such a wonderful group.

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My family has been an unfailing source of love and strength that has kept me on my feet when times were rough. And last but certainly not least, I would like to thank my husband, Michael, for his continuous support and encouragement. His humorous anecdotes kept me smiling throughout, and to him I say, "blah, blah, blah...laminin."
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CHAPTER 1

Introduction
Mechanisms of axonal guidance during pathfinding:

A functional nervous system is dependent upon the formation of appropriate synaptic connections by millions of neurons. During development, neuronal processes must grow through an ever-changing environment, often long distances, to reach their final target tissues where synaptic connections will be made. This presents an enormous task for each neuron, and is magnified by the variety of neuronal types and target tissues available. Due to the complex nature of this problem, it is feasible that several different mechanisms are utilized by neurons, possibly in distinct combinations, to provide guidance information.

The means by which growth cones choose the appropriate pathway have intrigued researchers for years, and are slowly becoming apparent. Many different types of experiments have been done to examine this issue, and have resulted in the production of a variety of hypotheses. One of the simplest hypotheses suggested that stereotropism, or mechanical guidance, was a necessary factor in directing neurons to their target tissues. This idea developed due to experiments in which neurons in vitro were observed to send neurites along scratches in a culture dish (Weiss 1941), grow preferentially along the path of least resistance, or were influenced by changes in surface contours (Horder and Martin 1979). A more specific rendition of this idea, called the blueprint hypothesis, suggested that spaces present in the neuroepithelial matrix prior to axonal ingrowth provided channels through which axons could be directed to their final destinations (Singer, Nordlander et al. 1979). This mechanism appears to play a major role in the outgrowth of spinal cord axons in the embryonic newt (Singer, Nordlander et al. 1979), and may have a partial role in directing retinal ganglion cell axons toward the optic stalk in chick (Krayanek and Goldberg 1981). The inherent weaknesses of this hypothesis are that it does not account for the majority
of pathfinding, since channels are not available to all axons, and no specific mechanism is provided to explain how axons are directed onto or off of major pathways.

Another hypothesis, called the chemoaffinity hypothesis, was developed by Sperry and colleagues in 1963 after examining the regeneration of retinal ganglion cell (RGC) axons in amphibians (Sperry 1963). When RGC axons were severed, they reinnervated the tectum in a precise manner such that neighboring cells in the retina innervated adjacent regions of the tectum. This innervation pattern resulted in a topographic map of the retina onto the tectum, and was presumably due to gradients of specific chemical labels to which the neurons responded. Therefore, the growth cones were thought to be guided toward their target cells in the tectum by specific chemotactic cues. Subsequent experiments by other investigators have supported the role of chemoaffinity (Walter, Kern-Veits et al. 1987; Fraser and Perkel 1990) and the establishment of gradients (Baier and Bonhoeffer 1992) for the initial growth of RGC axons to the tectum, but have also suggested that this is only one component of the pathfinding process. Upon reaching the tectum, the axonal terminals are refined and remodeled to result in the final pattern of specific connections (Yoon 1971; Schmidt 1978).

Several target tissues have been shown to produce gradients of soluble, diffusing signals that attract the appropriate growth cones from a distance in vitro. For example, the coculture of trigeminal neurons with their normal maxillary epithelium target in three dimensional collagen gels results in oriented outgrowth of the neurons toward the target (Lumsden and Davies 1983). The chemotactic factor in this interaction is distinct from nerve growth factor (NGF) (Lumsden and Davies 1983), and emanates only from the maxillary target epithelium and not from the mesenchyme tissue through which the axons normally grow en route to their target (Lumsden and Davies 1986). Other examples of chemotropism include the influence of floor plate cells on commissural
axon growth (Tessier-Lavigne, Placzek et al. 1988), and the role the basilar pons plays in attracting projections from the cerebral cortex (Heffner, Lumsden et al. 1990). Each of these targets produces its own distinct chemoattractant, and efforts are currently underway to characterize these molecules. Earlier experiments had provided evidence for a chemotropic role for NGF, in addition to its well-described neurotrophic role. Peripheral sympathetic fibers were observed to grow into the CNS in response to injection of NGF into the CNS (Menesini-Chen, Chen et al. 1978). In addition, dorsal root ganglion neurons in vitro have been shown to turn toward a locally applied source of NGF (Gundersen and Barrett 1979) although this was most likely due to the NGF becoming bound to the culture dish. Unfortunately, nonphysiological quantities of NGF were required to obtain such effects, and NGF is first expressed by target tissues at the time of growth cone arrival, not before (Davies, Bandtlow et al. 1987), suggesting that its main effects are on neuronal survival. The above data suggest that chemotropism may be a viable method of specific axon guidance in some cases, but certainly does not account for all pathfinding events.

Contact-mediated repulsive signals have also been described, and are thought to prevent growth cones from growing into inappropriate regions. The hallmark of these repulsive signals is the inducement of collapse, and often retraction, of growth cones (Keynes and Cook 1990; Walter, Allsopp et al. 1990; Davies and Cook 1991; Fawcett 1993). This type of mechanism was first described by Kapfhammer and Raper. Using a culture assay, they found that sympathetic growth cones collapsed upon contact with retinal neurites, and tended to avoid the neurites of different origin (Kapfhammer, Grunewald et al. 1986; Kapfhammer and Raper 1987). The converse case was also shown, suggesting the existence of two distinct labels, one associated with peripheral neurites and one associated with central neurites, that could be recognized by the growth cones during pathfinding (Kapfhammer and Raper 1987). In concert with this
idea, a glycoprotein isolated from brain called collapsin induces the collapse of peripheral sensory ganglion growth cones but not retinal ganglion cell growth cones (Luo, Raible et al. 1993). In addition, proteins isolated from CNS myelin have also been shown to inhibit neurite growth, especially during regeneration (Caroni and Schwab 1988).

Several in vivo examples of contact-mediated inhibition have been described. During normal development, motor and sensory growth cones traverse only the anterior portion of each somite, avoiding the posterior half (Keynes and Stern 1984; Keynes and Stern 1985). Glycoproteins isolated from the posterior half of somites induce growth cone collapse (Davies, Cook et al. 1990), and motoneurons actively avoid posterior somite cells in vitro (Oakley and Tosney 1993). These results suggest that growth cones are prevented from entering posterior somites due to inhibitory mechanisms, but are able to extend through anterior somites where these inhibitory cues are not present.

In the retinotectal system, retinal axons of temporal origin normally innervate anterior regions of the tectum and axons of nasal origin innervate posterior tectal regions (Gaze 1970). In culture, temporal retinal axons avoid growing on membranes derived from posterior tectum due to the presence of a 33 kDa inhibitory glycoprotein (Walter, Allsopp et al. 1990). In addition, retinal neurons of temporal origin, but not nasal origin, collapse when posterior tectal membranes are added to cultures of retinal cells (Cox, Muller et al. 1990). These results suggest that a repulsive mechanism could be partially responsible for guidance of retinal axons to their appropriate site of innervation in the tectum. Another group of molecules, proteoglycans, have been localized to several regions that axons avoid in vivo (Snow, Steindler et al. 1990; Oakley and Tosney 1991; Britts, Canning et al. 1992), and have been shown to inhibit neurite growth in vitro (Snow, Lemmon et al. 1990; Snow, Watanabe et al. 1991).
Proteoglycans may therefore act as another class of inhibitory molecules in vivo. Chemoattractants and contact-mediated repulsive mechanisms could potentially work in concert; one to attract the growth cones and the other to deflect them from inappropriate regions. Since these mechanisms only affect a subset of growth cones, they are probably highly specific and cannot account for all pathfinding in the nervous system.

A small group of studies have suggested that other less widely accepted mechanisms may also play a partial role in guidance. Examples of these include the influence of electrical fields, or galvanotropism, such that voltage gradients present in the embryo may provide guidance information (Jaffe and Poo 1979; Jaffe 1981). And more recently, locally applied neurotransmitters have been shown to induce growth cone turning (Zheng, Felder et al. 1994).

One of the more widely accepted ideas for explaining how growth cones choose pathways en route to their targets was the differential adhesiveness hypothesis. This idea developed from tissue culture studies using patterned substrates in which growth cones were shown to grow preferentially along the pathway of greatest adhesivity (Letourneau 1975; Letourneau 1975; Hammarback, McCarthy et al. 1988). Upon extrapolation to the embryo, the existence of a few growth-promoting molecules with different adhesivity properties could be presented in a variety of combinations to result in specific highways that the growth cones follow to their targets (Rutishauser 1986). Although this idea was attractive, it was limited in scope and did not explain why axons that originated in the same bundle were observed to diverge at specific choice points (Landmesser 1984). By reinvestigating this issue using molecules that growth cones normally encounter in the embryo, it was demonstrated that the level of adhesivity does not directly correlate with substrate choice by neurites (Gundersen 1987; Gundersen 1988; Lemmon, Burden et al. 1992). Therefore, these molecules may influence growth cones by other means, such as the production of intracellular signals (Doherty and
Walsh 1992; Doherty and Walsh 1994). In addition, it has become clear that many of these molecules promote neurite growth from specific types of neurons, suggesting that neurons may possess distinct complements of receptors for the available molecules in the embryo.

**Cell adhesion molecules have distinct roles during pathfinding in invertebrates:**

Genetic studies of less complex invertebrate systems such as *Drosophila* and *Caenorhabditis elegans* have shown that many cell adhesion molecules (CAMs) expressed on the surface of neurons and other cells act not only to promote adhesion and axonal growth, but provide directional cues as well (McIntire, Garriga et al. 1992; Bunch and Brower 1993). Many of these molecules belong to the Immunoglobulin Superfamily or Cadherin Family of adhesion molecules (see below), and are homologous to molecules isolated from vertebrates. Therefore, functional information acquired about these molecules from simpler organisms may provide insight into the role of homologous molecules in vertebrate pathfinding events.

Fasciclin II (fas II) is a transmembrane glycoprotein that has been identified in both grasshopper and *Drosophila* (Bastiani, Harrelson et al. 1987; Grenningloh, Rehm et al. 1991). It is a member of the Immunoglobulin Superfamily and has structural similarity with the vertebrate Neural Cell Adhesion Molecule (NCAM), but only possesses 19-23% amino acid homology with the various forms of NCAM that have been described (Harrelson and Goodman 1988; Grenningloh, Rehm et al. 1991). Also, it promotes homophilic adhesion (Grenningloh, Bieber et al. 1990). In *Drosophila*, fas II is distributed initially on two pioneering neurons that define the MPI neural pathway, and is later expressed by several other neurons that fasciculate along this pathway. In addition, fas II is subsequently expressed by neurons in many of the longitudinal and
commissural axon fascicles, as well as by a subset of motor and sensory neurons outside the central nervous system (Grenningloh, Rehm et al. 1991). *Drosophila* fas II mutants do not display a substantial alteration in the CNS, but do lack the MP1 pathway, suggesting that fas II acts as a neural recognition molecule in the MP1 pathway (Grenningloh, Rehm et al. 1991).

*Drosophila* neuroglial is a transmembrane glycoprotein that shares greater than 28% homology with the mouse neural adhesion molecule L1, and is a member of the Immunoglobulin Superfamily (Bieber, Snow et al. 1989). It occurs in two forms which differ in their cytoplasmic domains due to tissue-specific alternative splicing (Hortsch, Bieber et al. 1990). The long form is restricted in expression to neurons in the CNS and both neurons and support cells in the PNS, whereas the short form is expressed by a variety of non-neuronal cells (Bieber, Snow et al. 1989; Hortsch, Bieber et al. 1990). Neuroglial promotes homophilic adhesion when transfected into nonadhesive *Drosophila* S2 cells (Grenningloh, Bieber et al. 1990). Mutants die as embryos presumably due to an essential function of neuroglial in the formation of certain non-neuronal tissues such as trachea or hindgut (Bieber, Snow et al. 1989). The overall structure of the CNS and PNS appears to develop normally in these mutants though, suggesting that a redundant adhesion mechanism may compensate in the nervous system and result in normal development (Bieber, Snow et al. 1989).

Amalgam is another protein that has been isolated from *Drosophila* and shown to be a member of the Immunoglobulin Superfamily (Seeger, Haffley et al. 1988). Amalgam contains three immunoglobulin-like domains that share sequence similarity with several vertebrate proteins including NCAM, myelin associated glycoprotein, and human Tactile protein (Seeger, Haffley et al. 1988) (Wang, O'Farrell et al. 1992). During *Drosophila* embryogenesis, amalgam is expressed on the surface of neural cells and
other mesodermal cells, yet its function has not yet been elucidated (Seeger, Haffley et al. 1988).

Multiple proteins isolated from Drosophila have been indicated in the development of neuromuscular specificity (Nose, Vactor et al. 1992; Bunch and Brower 1993). Examples of these proteins include connectin (Nose, Mahajan et al. 1992), toll (Nose, Vactor et al. 1992), fasciclin III (Nose, Vactor et al. 1992; Bunch and Brower 1993) and neuromusculin (Kania, Han et al. 1993). Connectin protein contains ten leucine-rich repeats and is connected to the cell membrane by a phosphatidylinositol anchor (Nose, Mahajan et al. 1992). It acts as a homophilic adhesion molecule when transfected into Drosophila S2 cells. During embryonic development, connectin is expressed on eight abdominal muscles, the motoneurons that innervate them and a portion of glial cells along the pathway leading to the muscles, suggesting that it has a role in target recognition (Nose, Mahajan et al. 1992). The toll protein contains two leucine-rich repeats, a transmembrane domain and a region in its cytoplasmic domain with significant sequence homology to the vertebrate interleukin I receptor (Schneider, Hudson et al. 1991). Although the initial function of toll consists of transmission of a dorsoventral signal across the oocyte membrane (Anderson, Jurgens et al. 1985), it is later expressed on a subset of muscles and may be involved in motoneuron target recognition (Nose, Mahajan et al. 1992). Fasciclin III (fas III) is a transmembrane protein that exists as two forms with different cytoplasmic domain lengths due to alternative splicing (Grenningloh, Bieber et al. 1990). It possesses three divergent immunoglobulin-like domains and has been shown to participate in homophilic adhesion (Grenningloh, Bieber et al. 1990). In the CNS, fas III is expressed on a subset of commissural axon bundles (Patel, Snow et al. 1987), but in the periphery it is expressed by a subset of motoneurons that innervate fas III-expressing muscles (Halpern, Chiba et al. 1991). Therefore, it could be involved in guiding these
motoneurons to their appropriate target muscles. Neuromusculin is a member of the Immunoglobulin Superfamily that mediates homophilic cell adhesion (Kania, Han et al. 1993). The expression pattern of neuromusculin on peripheral neuronal precursors and muscles suggests that it may be involved in fasciculation or pathfinding (Kania, Han et al. 1993), although this has yet to be shown.

Four genes, longitudinals lacking (lola), longitudinals gone (logo), commissureless (comm) and roundabout (robo), were recently isolated from Drosophila, and shown to have distinctive roles in growth cone pathfinding (Seeger, Tear et al. 1993). In both lola and logo mutants, the longitudinal axon pathways are significantly reduced or lacking, while the commissural pathways appear relatively normal. The lola phenotype appears to be due to a stalling in the forward progress of the pCC, vMP2 and MP1 growth cones that form the MP1 pathway, although the glia along this pathway are in their normal position. The logo phenotype is partially due to defects in guidance along nerve root glia. In the comm mutant, the commissural axon pathways are lacking while the longitudinal pathways appear normal. The comm phenotype is not due to a lack of midline cells or a change in their fate, but rather is due to the loss of a molecule that attracts specific neurons to the midline of the CNS. In the robo mutant, the pCC, vMP2 and MP1 axons which normally extend along one side of the CNS are misdirected and grow along the midline. The neuronal cell fates and midline glial cells appear normal in robo mutants, suggesting that the robo phenotype is due to the lack of a repulsive signal that normally prevents pCC, vMP2 and MP1 from entering the midline area. The protein products of these four genes have yet to be characterized.

Fasciclin I (fas I) is a glycoprotein that occurs either attached to the cell membrane by a GPI-anchor or is secreted (Hortsch and Goodman 1990). It contains four homologous repeats that do not share homology to other sequenced proteins (Zinn, McAllister et al. 1988), although it has been shown to act as a homophilic adhesion
molecule that mediates cell sorting (Elkins, Hortsch et al. 1990). Fas I is localized to fasciculating axons, with strongest expression in the commissural regions, but is also expressed on a variety of non-neuronal cells outside the CNS (Bastiani, Harrelson et al. 1987; McAllister and Goodman 1992). Protein null mutations in fas I do not result in gross defects in morphogenesis of the nervous system (Elkins, Zinn et al. 1990). However, embryos doubly mutant for fas I and the Abelson tyrosine kinase (abl) display severe defects in CNS axon pathways, especially in regions where these two proteins normally overlap, such as the commissural tracts (Elkins, Zinn et al. 1990). The mutant phenotype is due to a disruption in growth cone guidance for at least some neurons, such as RP1, and provides evidence for interaction between an adhesion molecule and intracellular signal transduction pathways (Elkins, Zinn et al. 1990). This type of interaction has recently been implicated between vertebrate adhesion molecules and intracellular signaling effectors (see signaling section below; Doherty and Walsh 1992; Doherty and Walsh 1994), and lends credence to the idea that information obtained from Drosophila may be directly relevant to vertebrate systems.

**Cell adhesion molecules and extracellular matrix molecules isolated from vertebrates:**

To date, a large and diverse group of CAMs and extracellular matrix (ECM) molecules have been isolated from developing vertebrate embryos. Many of these purified molecules have been shown to promote neurite growth when used as a culture substrate in vitro, and therefore may have a similar function in vivo. These molecules could provide the permissive highways upon which growth cones grow passively, or could be instructive in nature to direct growth cones specifically at choice points. Based upon structural features, neural CAMs are divided into two families; the Immunoglobulin Superfamily and the Cadherin family of adhesion molecules. The
molecules present in the ECM are principally recognized by cellular integrin receptors, a type of substrate adhesion molecule (SAM). The following is a description of the CAM and ECM molecules that have been isolated from vertebrates.

**Adhesion molecules of the Immunoglobulin Superfamily:**

Members of the Immunoglobulin Superfamily have several distinguishing features. They all share regions homologous to immunoglobulin-like (Ig) V or C domains, yet a large variation in overall structure and function exists between molecules (Yoshihara, Oka et al. 1991). Many of these molecules are expressed in the nervous system of developing vertebrates, and are thought to be involved in axon guidance as well as other morphoregulatory interactions. In addition, members of this family promote cell-cell adhesion in a calcium independent manner. The Immunoglobulin Superfamily has been subdivided into three classes based upon structural features of its individual members (Brummendorf and Rathjen 1993).

**Class I:**

The first class consists of molecules having only Ig domains. Members of this class include Po (Schneider-Schaulies, Brunn et al. 1990), Thy-1 (Yoshihara, Oka et al. 1991), MRC OX-2 (Clark, Gagnon et al. 1985), CD4 (Gorman, Tourvieille et al. 1987), Myelin Associated Glycoprotein (MAG) (Salzer, Holmes et al. 1987), Schwann cell myelin protein (SMP) (Dulac, Tropak et al. 1992), telencephalin (TLN) (Yoshihara, Oka et al. 1994), basigin/HT7 (Seulberger, Lottspeich et al. 1990), carcinoembryonic antigen (CEA) (Beauchemin, Benchimol et al. 1987), gicerin (Taira, Takaha et al. 1994), the FGF receptor (Yazaki, Fujita et al. 1993) and DM-GRASP/SCI/JC7 (Burns, Kannen et al. 1991; Tanaka, Matsui et al. 1991; El-Deeb, Thompson et al. 1992). Thy-1 and MRC OX-2 were originally isolated from thymus,
but have also been found in brain where they are neuron-specific (Morris 1985; Morris and Beech 1987). Thy-1 is a small glycoprotein consisting of a single V-like Ig domain attached to the membrane by a glycosyl phosphatidylinositol (GPI) anchor (Yoshihara, Oka et al. 1991). MRC OX-2 is an integral membrane protein with two Ig domains, one V-like and one C2-like domain, and a short cytoplasmic tail (Clark, Gagnon et al. 1985). Of interest is that a reciprocal pattern of expression for these molecules is observed in the developing cerebellum, such that OX-2 expression is strongest during axonogenesis whereas Thy-1 expression is upregulated during dendritic growth (Morris and Beech 1987). Therefore, these molecules appear to have distinct, specific functions during brain development.

Another molecule initially isolated from the immune system is CD4. This molecule is expressed on the surface of T-helper cells, and is involved in the recognition of class II molecules of the major histocompatibility complex (MHC) on antigen presenting cells (Doyle and Strominger 1987). CD4 isolated from brain is an integral membrane glycoprotein with a short cytoplasmic region and three Ig domains, two V-like and one C2-like (Gorman, Tourvieille et al. 1987). It is thought that CD4 acts as the cellular receptor for the human immunodeficiency virus (HIV), and therefore the subset of neurons and glial cells in the CNS that express CD4 may facilitate HIV infection of the brain (Funke, Hahn et al. 1987).

Basigin/HT7 possesses two C2-like Ig domains, a transmembrane region and a short cytoplasmic region (Seulberger, Lottspeich et al. 1990). It is expressed exclusively on the surface of brain endothelial cells and is thought to participate in the formation of the blood-brain barrier (Seulberger, Lottspeich et al. 1990). Another molecule, CEA, is produced by cells of several different carcinomas, and is involved in homotypic cell interactions (Benchimol, Fuks et al. 1989). Its molecular structure consists of seven Ig C2-like domains, a transmembrane region and a short cytoplasmic
tail (Beauchemin, Benchimol et al. 1987). To date, the function of CEA in the nervous system has been poorly characterized. Gicerin is a glycoprotein that contains five Ig domains, two V-like and three C2-like domains, a transmembrane region and a cytoplasmic tail with several potential phosphorylation sites (Taira, Takaha et al. 1994). Gicerin acts as the heterophilic binding partner of Neurite Outgrowth Factor (NOF), an ECM glycoprotein that induces neurite outgrowth from several neuronal cell types (Miki, Hayashi et al. 1981; Hayashi and Miki 1985; Kato, Taniura et al. 1992). Being expressed transiently in the developing brain, gicerin may regulate neuronal process extension and synapse formation.

The FGF receptor is an interesting member of the Immunoglobulin Superfamily. Its molecular structure consists of three Ig domains, a transmembrane region and two tyrosine kinase domains on the cytoplasmic portion of the receptor (Yazaki, Fujita et al. 1993). Subsequent to binding FGF, the receptor dimerizes and autophosphorylates, resulting in the induction of mitogenic responses by cells (Schlessinger and Ullrich 1992). Recently, activation of the FGF receptor has been implicated in neurite outgrowth via a pathway that involves L- and N-type calcium channels, a tyrosine kinase and arachidonic acid (Williams, Furness et al. 1994). Other recent studies have suggested that the FGF receptor may also interact with certain CAMs in a cis fashion to induce neurite outgrowth (Williams, Furness et al. 1994).

Telencephalin (TLN) is an ICAM-related glycoprotein found exclusively on the somas and dendrites of neurons in the telencephalon (Mori, Fujita et al. 1987; Oka, Mori et al. 1990). It consists of nine Ig domains, five of which contain four cysteine residues, a transmembrane region and a short cytoplasmic tail (Yoshihara, Oka et al. 1994). The main function of TLN appears to be dendritic-dendritic interactions during process outgrowth, and an involvement in the segmental organization of the brain (Yoshihara, Oka et al. 1994). DM-GRASP/SC1/JC7 is expressed on a subset of
neurons in chick, acts as a homophilic cell adhesion molecule (Tanaka, Matsui et al. 1991) and supports neurite outgrowth when used as a substrate in vitro (Burns, Kannen et al. 1991; El-Deeb, Thompson et al. 1992). It contains five Ig domains, two that are V-like and three that are C-like, a transmembrane region and a relatively short cytoplasmic region (Burns, Kannen et al. 1991; Tanaka, Matsui et al. 1991). Studies have suggested that it may be involved in neurite growth and fasciculation in vivo to result in formation of specific nerve bundles such as the lateral motor column (Tanaka, Matsui et al. 1991).

Three of the molecules in this class, Po, SMP, and MAG are expressed on myelinating glial cells and are thought to be involved in the process of myelination. Po is a small glycoprotein with only one Ig V-like domain, a transmembrane region and a short cytoplasmic tail (Lemke and Axel 1985). Its expression is restricted to the surface of Schwann cells where it is one of the most abundant proteins, constituting greater than fifty percent of the total protein on the peripheral myelin sheath (Ishaque, Roomi et al. 1980). Po binding occurs homophilically, and is thought to be involved in the compaction of the myelin sheath (Filbin, Walsh et al. 1990), but has also been shown to support neurite outgrowth (Schneider-Schaulies, Brunn et al. 1990). MAG is found in the membrane of both myelin-forming Schwann cells and oligodendrocytes, and was originally thought to be necessary during initiation of myelination (Poltorak, Sadoul et al. 1987), but recent examination of MAG-deficient mice has shown that this is not the case (Li, Tropak et al. 1994; Montag, Giese et al. 1994). Two forms of MAG have been isolated (Salzer, Holmes et al. 1987), small MAG and large MAG, and each form has been localized to the CNS and PNS (Pedraza, Frey et al. 1991). Both forms have five Ig C2-like domains and span the membrane, but their cytoplasmic region differs due to alternative splicing (Salzer, Holmes et al. 1987). SMP is an integral membrane protein with five Ig domains, four of which are C2-like, and a short cytoplasmic region.
(Dulac, Tropak et al. 1992). SMP expression occurs on the surface of myelinating and non-myelinating Schwann cells and oligodendrocytes (Dulac, Cameron-Curry et al. 1988), as well as on glial precursors (Dupin, Baroffio et al. 1990). The function of SMP has been poorly characterized to date.

**Class II:**

The presence of Ig domains and fibronectin type III domains (FNIII) defines those molecules in the second class of this family. Members of this class include NCAM (Cunningham, Hemperly et al. 1987), L1 and its chicken homologues (NgCAM/8D9/G4, Bravo/NrCAM, and neurofascin) (Lemmon and McLoon 1986; Rathjen, Wolff et al. 1987; Burgoon, Grumet et al. 1991; Grumet, Mauro et al. 1991; Kayyem, Roman et al. 1992; Volkmer, Hassel et al. 1992), F11/F3 (Brummendorf, Wolff et al. 1989; Gennarini, Cibelli et al. 1989), Axonin-1/TAG-1 (Furley, Morton et al. 1990; Zuellig, Rader et al. 1992), Deleted in Colon Cancer (DCC) (Fearon, Cho et al. 1990) and a heterogeneous group of receptor protein tyrosine phosphatases (Alexander 1990). Most of the current molecules in this family promote neurite outgrowth.

NCAM represents a unique subclass within this group due to its structure and the large number of alternatively spliced variants. It has five Ig C2-like domains, two FNIII-like domains, and the presence of either a cytoplasmic domain or membrane linkage by a GPI anchor (Cunningham, Hemperly et al. 1987). It is interesting that a carbohydrate, polysialic acid, is associated with the embryonic form of NCAM and has been shown to alter its binding properties and ability to promote neurite outgrowth (Crossin, Edelman et al. 1984; Acheson, Sunshine et al. 1991; Zhang, Miller et al. 1992). NCAM binding occurs homophilically, and also heterophilically with heparin (Rutishauser, Hoffman et al. 1982; Cole and Akeson 1989). The alteration in the
amount and form of NCAM expressed in the developing embryo suggests that it may be necessary for normal morphogenesis (Rutishauser 1983; Chuong and Edelman 1984; Edelman 1988), although recent knockout studies have shown that this function may be restricted to specific regions of the embryo (Tomasiewicz, Ono et al. 1993).

L1-related molecules comprise the second subclass in this group. They possess six Ig C2-like domains, five FNIII-like domains, a transmembrane region and a highly conserved cytoplasmic region (Lemmon and McLoon 1986; Rathjen, Wolff et al. 1987; Burgoon, Grumet et al. 1991; Grumet, Mauro et al. 1991; Kayyem, Roman et al. 1992; Volkmer, Hassel et al. 1992). L1 is mainly confined to axons in the nervous system where it is thought to play an important role in axonal fasciculation (Lemmon and McLoon 1986), yet one L1-related molecule, NgCAM, has also been suggested to promote binding between neurons and glial cells (Grumet and Edelman 1984; Grumet, Hoffman et al. 1984). L1-induced cell adhesion occurs in a homophilic fashion (Lemmon, Farr et al. 1989), and also occurs heterophilically with Axonin-1 (Sonderegger and Rathjen 1992), F11 (Brummendorf, Hubert et al. 1993), and laminin (Grumet, Friedlander et al. 1993). The L1-related molecule NrCAM has also been shown to interact heterophilically with F11 (Morales, Hubert et al. 1993).

The third subclass of molecules contains F11/F3 and Axonin-1/TAG-1, molecules with six Ig C2-like domains, four FNIII-like domains and a GPI linkage to the membrane (Brummendorf, Wolff et al. 1989; Gennarini, Cibelli et al. 1989; Furley, Morton et al. 1990; Zuellig, Rader et al. 1992). Although these molecules occur both as membrane-bound and soluble forms, the majority of F11/F3 is membrane-bound whereas Axonin-1/TAG-1 mainly occurs as a soluble form (Stoeckli, Lemkin et al. 1989; Moss and White 1992). F11/F3 has been shown to mediate only heterophilic interactions with NgCAM, restrictin (Brummendorf, Hubert et al. 1993), and NrCAM/Bravo (Morales, Hubert et al. 1993). Axonin-1 binds in both a homophilic
fashion (Rader, Stoeckli et al. 1993) and heterophilically with NgCAM, but requires
the interaction with NgCAM for the promotion of neurite outgrowth (Kuhn, Stoeckli et
al. 1991). TAG-1 also binds homophilically, but requires the cooperation of L1 and
the β1 integrin for promotion of neurite outgrowth (Felsenfeld, Hynes et al. 1994).

The fourth subclass of molecules, represented by DCC, has four Ig C2-like
domains, one FNIII domain and a transmembrane region (Fearon, Cho et al. 1990).
The expression of DCC, a putative tumor suppressor, is altered in several cancerous
tissues including gliomas (Scheck and Coons 1993). When transfected into
fibroblasts, DCC promotes neurite outgrowth from PC12 cells (Pierceall, Cho et al.
1994), suggesting that it could promote axonal growth in vivo.

The last subclass of molecules contains a heterogeneous group of receptor protein
tyrosine phosphatases (RPTPs). These molecules were placed into this class due to
their extracellular domain properties, although their phosphatase domains are unique.
Several members of this group have combinations of Ig C2-like domains, FNIII
domains and/or carbonic anhydrase domains in a variety of different structural
arrangements (Alexander 1990; Barnea, Silvennoinen et al. 1993). Expression in
multiple tissue types including nervous tissue suggests diverse functions for these
molecules.

Class III:

The last class of Ig superfamily molecules is defined by the presence of other
domains besides Ig and FNIII domains, such as EGF repeats, hyaluronic acid binding
domains or tyrosine kinase domains. Members of this class include the neurotrophin
receptors (Barbacid, Lamballe et al. 1991; Schneider and Schweiger 1991), neuregulin
(Wen, Peles et al. 1992) and neurocan (Rauch, Karthikeyan et al. 1992). The
molecular structure of the trk family of neurotrophin receptors contains two Ig C2-like
domains and a tyrosine kinase domain (Barbacid, Lamballe et al. 1991; Schneider and Schweiger 1991). These molecules are involved in the transmembrane signaling of growth factor binding, and may also function as cell adhesion molecules in the nervous system (Schneider and Schweiger 1991). Neurocan is a secreted chondroitin sulfate proteoglycan that possesses one Ig C-like domain, two EGF repeats and a lectin-like domain (Rauch, Karthikeyan et al. 1992). Although neurocan inhibits the homophilic interaction between NCAM or NgCAM (Friedlander, Milev et al. 1994), it has not been shown to act as a classic cell adhesion molecule. Neuregulin is the putative ligand of the c-neu receptor tyrosine kinase and is involved in the induced differentiation of several cell types (Wen, Peles et al. 1992). The form of neuregulin expressed in the brain contains one Ig domain and one EGF repeat, as well as a transmembrane and cytoplasmic domain (Meyer and Birchmeier 1994), although its function in the nervous system has not yet been characterized. To date, none of the molecules in this class have been demonstrated to promote neurite outgrowth directly, but they could influence neuronal behavior in some manner that has yet to be determined.

The Cadherin Family of adhesion molecules:

Cadherins are cell surface adhesion molecules that bind by a calcium dependent mechanism. The characteristic features of cadherins include an extracellular domain with several repeated sequences of three to five amino acids, four cysteines adjacent to the transmembrane domain, and a cytoplasmic domain that, for some cadherins, has been shown to interact with catenin proteins (Takeichi 1990; Takeichi, Hirano et al. 1992; Gumbiner 1993; Kemler 1993). In the vertebrate nervous system, the best-characterized cadherins are N-cadherin, R-cadherin, B-cadherin and T-cadherin. N-cadherin is expressed by neuronal and non-neuronal cells and may play a role in heterotypic cell-cell interactions (Drazba and Lemmon 1990; Letourneau, Shattuck et al.
1990; Letourneau, Roche et al. 1991) as well as in morphoregulatory events in the embryo (Hatta, Takagi et al. 1987). It is a potent inducer of neurite outgrowth when used as a substrate (Bixby and Zhang 1990), and has been indicated as a guidance factor for optic axons (Matsunaga, Hatta et al. 1988). The second cadherin expressed in nervous tissue, R-cadherin, was initially isolated from the chick retina (Inuzuka, Miyatani et al. 1991), but was later localized to a variety of tissues of nervous and mesodermal origin (Inuzuka, Redies et al. 1991; Redies, Inuzuka et al. 1992). R-cadherin shares a 74% homology at the amino acid level with N-cadherin, with 72% identity in the extracellular domain and 85% identity in the cytoplasmic domain (Inuzuka, Miyatani et al. 1991). N-cadherin and R-cadherin bind in a homophilic and heterophilic fashion (Takeichi 1990; Inuzuka, Miyatani et al. 1991), suggesting possible overlapping and distinct functions. B-cadherin is most similar to E- and P-cadherin in all regions of its extracellular, transmembrane and cytoplasmic domains (Napolitano, Venstrom et al. 1991). It binds in a homophilic fashion and is expressed in both neuronal and non-neuronal tissues (Murphy-Erdosh, Napolitano et al. 1994), although its function during development has not yet been directly shown.

T-cadherin shares 47% homology with N-cadherin in the extracellular domain, but lacks the conserved cytoplasmic domain, and is attached to the cell membrane by a glycosyl phosphatidylinositol anchor (Ranscht and Dours-Zimmerman 1991). Although T-cadherin is not limited to the nervous system, it is expressed in a temporally and spatially restricted pattern that is consistent with a role in axon guidance (Ranscht and Dours-Zimmerman 1991). T-cadherin binds in a calcium-dependent, homophilic manner (Vestal and Ranscht 1992), but has several properties that are distinct from the classic cadherins. For example, both the uncleaved and mature forms of T-cadherin are expressed on the surface of transfected CHO cells, T-cadherin is more resistant to proteolytic cleavage in the absence of calcium, and is not concentrated
in cell-cell contacts between transfected cells (Vestal and Ranscht 1992). A T-cadherin isoform, T-cadherin 2, was recently characterized and shown to have an extended carboxy terminal region, but maintains similar functional properties as T-cadherin (Sacristan, Vestal et al. 1993). In addition to those described above, several other cadherins have been isolated but have not yet been thoroughly characterized (Suzuki, Sano et al. 1991; Sano, Tanihara et al. 1993). The overlapping expression of multiple cadherins within tissues and individual neurons suggests that cell adhesion within a developing embryo involves complex interactions between several cadherins (Sacristan, Vestal et al. 1993).

Molecules of the extracellular matrix (ECM):

The extracellular matrix (ECM) consists of a diverse group of secreted glycoproteins and other molecules that are immobilized on the surface of cells. Many of these molecules have specific functions during nervous system development, such as a role in neural crest migration, cellular differentiation, axonal guidance and synapse formation (Reichardt and Tomaselli 1991; Hynes and Lander 1992). Laminin is an ECM molecule that has been studied extensively due to its diverse roles in developmental processes and its ability to promote neurite outgrowth from a wide variety of neurons (Kleinman, Sephel et al.; Edgar 1991). It is a large glycoprotein consisting of three separate chains, designated A, B1 and B2, held together in a cruciform structure by disulfide bonds (Kleinman, Sephel et al.; Reichardt and Tomaselli 1991). Each of these chains has multiple homologous domains, with putative globular domains at their amino terminal ends and several EGF-like repeats. In addition, the A chain is extended at its carboxy terminal region by several putative globular domains not present in the B chains. Isolation of the major proteolytic fragments of laminin has allowed characterization of a number of integrin binding sites
as well as neurite outgrowth-promoting regions (Tashiro, Sephel et al. 1989; Hall, Reichardt et al. 1990; Tomaselli, Hall et al. 1990). In the developing embryo, laminin is expressed on neural cell membranes and in the interstitial ECM, but later becomes incorporated into basement membranes (Edgar 1991). Several laminin variants have been described (Hunter, Shah et al. 1989; Ehrig, Leivo et al. 1990; Engvall, Earwicker et al. 1990; Paulsson, Saladin et al. 1991). One of these, merosin, was initially isolated from placenta and is similar in structure to laminin, except that the laminin A chain is replaced by the merosin M chain (Ehrig, Leivo et al. 1990). Like laminin, merosin promotes cell attachment and neurite outgrowth via a β1 containing integrin (Cohen and Johnson 1991; Engvall, Earwicker et al. 1992). S-laminin is a laminin variant that is similar to the B1 chain in structure (Hunter, Shah et al. 1989). It is expressed principally in the synaptic cleft of the neuromuscular junction (Hunter, Shah et al. 1989), and promotes cell adhesion by a leucine-arginine-glutamate-dependent mechanism (Hunter, Cashman et al. 1991).

Fibronectin is a secreted ECM protein that consists of a single molecule with a number of structural repeats (denoted FN1, FNII, and FNIII-like repeats), and a RGDS site that functions in cell attachment (Reichardt and Tomaselli 1991). The FNIII domains are differentially spliced (Hynes 1985), resulting in distinct forms of fibronectin with specific spatial and temporal expression patterns (Pagani, Zagato et al. 1991). Of interest, the V25-variant which has been implicated in neurite outgrowth is highly expressed in the developing brain and is later down-regulated (Pagani, Zagato et al. 1991). In the developing cortex, fibronectin is present on the surfaces of radial glia in regions of neuronal migration and axonal growth (Sheppard, Hamilton et al. 1991). Similar expression patterns in other regions of the developing embryo suggest that fibronectin may play a partial role in the migration and growth of several neuronal types.
One of the major constituents of the ECM is collagen, and greater than twelve
distinct collagens have been described (Reichardt and Tomaselli 1991). The structure
of collagen consists of multiple GXY repeats, a FNIII domain, and von Willebrand
type A repeats (Letourneau, Condic et al. 1994). The NC1 domain of type IV collagen
has been shown to promote neurite outgrowth via an integrin mediated mechanism
(Lien, Higgins et al. 1991), and expression in several sites within the nervous system
suggest that collagen may be involved in neuronal adhesion and axonal extension in
vivo (Shiga and Oppenheim 1991; Reichardt, Bossy et al. 1992).

Several proteoglycans have been observed in the developing nervous system, and
have been implicated in the regulation of multiple functions including cell-cell adhesion,
cell migration, neurite elongation, and differentiation (Hockfield 1990). Examples of
these include heparin sulfate proteoglycan, chondroitin sulfate proteoglycan, dermatan
sulfate proteoglycan, perlecan and clausrin (Letourneau, Condic et al. 1992).
Proteoglycans can be expressed on the surface of cells where they act as receptors for
ECM components (see below) (Hoffman, Crossin et al. 1988) and also bind growth
factors (Thiery and Boyer 1992). These molecules consist of a core protein with
multiple glycosaminoglycan chains (Hockfield 1990; Reichardt and Tomaselli 1991). It
is thought that the diverse functions of proteoglycans are dependent upon the molecules
with which they interact, their spatial and temporal expression pattern, and the the
different responses of specific cell types to the proteoglycan subunits (Letourneau,
Condic et al. 1992).

Thrombospondin is a large, trimeric glycoprotein that has been shown to promote
and inhibit cell migration, mediate neurite outgrowth, and has both adhesive and anti-
adhesive functions (reviewed in (Bornstein 1992). This diversity in function is due to
its ability to interact with several different cell surface receptors, such as heparin sulfate
proteoglycans and the αvβ3 integrin (Frazier 1991). Differential tissue-specific
expression of the three structurally-related forms of thrombospondin suggest distinct functions for each (Iruela-Arispe, Liska et al. 1993). In its molecular structure, thrombospondin contains a heparin binding domain as well as EGF-like and Ca\(^{2+}\) binding repeats (Frazier 1991; Bornstein 1992).

Tenascin is a glia-derived ECM glycoprotein that is expressed at high levels in the nervous system (Erickson and Bourdon 1989). It is a six-armed hexabrachion with multiple EGF-like and FN III-like repeats that occurs in several forms due to alternative splicing (Saga, Tsukamoto et al. 1991). An interesting feature of tenascin is that both adhesive and anti-adhesive regions have been localized to the glycoprotein (Spring, Beck et al. 1989; Prieto, Andersson-Fisone et al. 1992), suggesting that it may have multiple roles in the nervous system. Of interest, tenascin promotes neurite outgrowth from a subset of neurons (Chuong, Crossin et al. 1987; Taylor, Pesheva et al. 1993), but also is expressed in regions where cytoarchitectonic boundaries exist (Steindler, Cooper et al. 1989). A family of tenascin-like glycoproteins have been described that share similar functional properties with tenascin, but consist of dimeric or trimeric armed rods (Pesheva, Spiess et al. 1989; Fuss, Wintergerdt et al. 1993).

Restrictin is a neural extracellular matrix protein isolated from chick that has been shown to associate with the cell surface protein F11 (Brummendorf, Hubert et al. 1993). It has substantial sequence similarity with tenascin, and has been shown to form oligomeric structures (Norenberg, Wille et al. 1992). In addition, restrictin promotes cell attachment in vitro (Rathjen, Wolff et al. 1991), and therefore may act as an adhesive molecule in vivo.

**Cellular recognition of ECM molecules:**

The majority of cellular receptors for ECM molecules have been isolated from non-neuronal cells such as fibroblasts and platelets, but many of these receptors have also
been localized to neurons and glial cells (Boyczko and Horwitz 1986; Cohen, Burme et al. 1987; Hall, Neugebauer et al. 1987; Tawil, Houde et al. 1990). Integrins are the primary cellular receptors for ECM components, consisting of noncovalently linked αβ heterodimers which form a membrane-spanning complex (Sonnenberg 1993). The globular extracellular domain of the integrin complex binds to its ECM ligand in a specific fashion that is dependent upon the presence of divalent cations (Marlin and Springer 1987; Gailit and Ruoslahti 1988). The ligand binding specificity and affinity of integrins is also regulated by post-translational modifications of the cytoplasmic domains (Hemler, Kassner et al. 1992; Hynes 1992).

A large number of α and β subunits have been characterized, and the pairing of certain α and β subunits results in the formation of integrins with distinct ligand specificity (Hynes 1987; Reichardt and Tomaselli 1991; Sonnenberg 1993). Of the available β subunits, only β₁, in conjunction with multiple α subunits, has been directly shown to play a functional role in neuronal interaction with ECM molecules (Tawil, Houde et al. 1990; Tomaselli, Hall et al. 1990; Toyota, Carbonetto et al. 1990; Tomaselli, Doherty et al. 1993). Upon the surface of an individual cell, multiple integrins may be expressed to allow for recognition of several different ECM molecules (Reichardt and Tomaselli 1991; Hynes 1992). In addition, a subset of integrins have been shown to interact heterophilically with members of the Immunoglobulin Superfamily for heterotypic cell-cell adhesion (Springer 1990) or the promotion of neurite outgrowth (Felsenfeld, Hynes et al. 1994).

Another molecule that has been identified as a receptor for the ECM is the hyaluronic-acid-binding protein, H-CAM (also called Hermes antigen or CD44). It is a transmembrane receptor that has been shown to interact with both ECM and cell surface-associated ligands (John, Meyer et al. 1990; Reichardt and Tomaselli 1991). H-CAM has been localized to CNS glia and Schwann cells (Picker, Nakache et al. 

...
1989), although it is not expressed by adult neurons. In the developing embryo, hyaluronic acid is present in several migratory pathways, including those used by neural crest cells where it is necessary for migration (Perris and Johansson 1990). Therefore, the interaction between H-CAM and hyaluronic acid may be necessary for normal migration of multiple cell types during development.

Proteoglycans are a third type of receptor for ECM molecules. One of these, syndecan, is a transmembrane proteoglycan that binds ECM glycoproteins via its glycosaminoglycan chains (Saunders, Jalkanen et al. 1989). Syndecan mediates the binding of multiple cell types to collagens and fibronectin (Saunders, Jalkanen et al. 1989; Reichardt, Bossy et al. 1990) and has also been shown to bind growth factors (Bernfield and Sanderson 1990). A large number of other, distinct proteoglycans are expressed in the developing mammalian brain (Herndon and Lander 1990), and may also be involved in ECM interactions.

Evidence for cis and trans interactions:

Trans interactions are defined as the binding that occurs between CAMs on two apposed cell surfaces. This type of interaction includes the homophilic and heterophilic binding events described in the three previous sections. Recently, evidence has been presented in support of cis interactions, or the interaction between two CAMs in the plane of the membrane on the same cell surface, for regulation of CAM function. CAM-coated bead aggregation assays (Kadmon, Kowitz et al. 1990) and chemical crosslinking studies (Simon, Klinz et al. 1991) showed that an assisted homophilic interaction occurs, such that a cis interaction between L1 and NCAM results in enhanced L1 binding between two apposed surfaces. This interaction appears to be carbohydrate dependent (Kadmon, Kowitz et al. 1990). Another type of cis interaction has been demonstrated for Ng-CAM and Axonin-1, and appears to potentiate neurite
outgrowth on Ng-CAM or Axonin-1 substrates (Stoeckli, Ziegler et al. 1993). An important cis interaction is the dimerization of FGF receptors that occurs subsequent to the binding of FGF, and results in autophosphorylation of defined tyrosine residues (Schlessinger and Ullrich 1992). Recently, it has been suggested that the FGF receptor may form a heterodimer in a cis interaction between L1, N-cadherin or NCAM to initiate the intracellular signals that elicit neurite outgrowth (Williams, Furness et al. 1994). Therefore, it seems that CAM-CAM interactions are no longer simple binding events, but instead CAMs may participate in a variety of complex interactions that could potentially produce very different recognition properties and intracellular responses.

**Intracellular signals induced by CAM or SAM binding:**

Recently, several laboratories have shown that the binding of CAMs on the surface of cells results in the activation of intracellular signal cascades (reviewed in (Doherty and Walsh 1992; Doherty and Walsh 1994). For example, the addition of purified CAMs or antibodies directed against the CAMs results in changes in intracellular pH, Ca\(^{2+}\), IP\(_2\) and IP\(_3\) levels (Schuch, Lohse et al. 1989), although some of these effects are cell specific (Halbach, Taylor et al. 1992). Doherty and colleagues have provided evidence suggesting that a G protein-dependent activation of Ca\(^{2+}\) channels may be involved in neurite outgrowth on N-cadherin, L1 or NCAMexpressing 3T3 cells, but not on laminin (Doherty, Ashton et al. 1991; Sařell, Walsh et al. 1992; Williams, Doherty et al. 1992). An erbstatin-sensitive tyrosine kinase (Williams, Walsh et al. 1994), and consequently arachidonic acid (Williams, Walsh et al. 1994), also appear to be involved in the upstream activation of the calcium channels for neurite outgrowth. Of interest is that neurite outgrowth stimulated by FGF appears to utilize the same second messenger pathway as that stimulated by the CAMs (Williams, Furness et al. 1994). These results, and others (Williams, Furness et al. 1994), suggest that the FGF
receptor may somehow interact with the CAMs, most likely in a cis fashion, to generate a signal for neurite outgrowth.

Several tyrosine kinases and phosphatases have been localized to growth cones of early embryos and implicated in growth cone function (Bixby and Jhabvala 1993). Cerebellar neurons from src mice display impaired neurite outgrowth on L1 but not laminin, suggesting a partial role for tyrosine phosphorylation via pp60⁹⁵-src in L1-mediated axonal growth (Ignelzi, Miller et al. 1994). In further support, two distinct protein kinase activities that are associated with and specifically phosphorylate L1 have been described (Sadoul, Kirchhoff et al. 1989; Schaefer, Wong et al. 1993). Tyrosine phosphorylation appears to be involved in integrin-mediated binding of ECM components by several non-neuronal cell types as well (Romer, Burrage et al. 1992). In contrast with these results, Bixby and Jhabvala showed that inhibitors of protein tyrosine kinases potentiated neurite outgrowth on L1, N-cadherin and several ECM molecules (Bixby and Jhabvala 1992). In addition, the triggering of L1 or NCAM on the surface of growth cone particles inhibited the pp60⁹⁵-dependent phosphorylation of tyrosyl residues on a subset of tubulin (Atashi, Klinz et al. 1992). It is interesting that inhibition of protein kinase C (PKC) prevents initial neurite outgrowth on laminin, fibronectin and collagen but not on L1 or N-cadherin, suggesting that integrin mediated signaling utilizes distinct signaling pathways (Bixby and Jhabvala 1990). It is clear that the binding of CAMs and SAMs results in the generation of intracellular signals that are necessary for neurite outgrowth (Doherty and Walsh 1994). Distinct growth rates, neuronal morphology and growth cone morphology are observed on CAMs and ECM molecules (Lemmon, Burden et al. 1992; Payne, Burden et al. 1992), suggesting that other, as yet undefined, signaling mechanisms may regulate these properties. Together, these results support that cellular interaction with CAMs and ECM molecules
produces a complex array of intracellular signal(s) that may play a role in diverse cellular responses.

The above studies have provided important information about how various CAMs and ECM molecules influence populations of neurons. In addition, this type of information provides clues to the function of these molecules in vivo. Within the following chapters, the results of several experiments that collectively focus on how CAMs and ECM molecules affect growth cone motility and behavior are presented. Chapter 2 describes the ability of several substrate-bound CAMs and ECM molecules to affect multiple growth parameters such as growth cone adhesivity and neurite selectivity. The results of these experiments have supported that substrate adhesivity is not a principal factor in axonal pathfinding. Chapter 3 describes experiments in which growth cones were examined with timelapse videomicroscopy as they interacted with a sharp border between substrates. The results from these experiments indicate that growth cone morphology and behavior are influenced by substrate-bound CAMs and ECM molecules, and suggest that interaction with CAMs and ECM molecules may result in the generation of distinct intracellular signals that mediate different behavioral responses of the growth cone. In Chapter 4, the distribution of cytoskeletal elements in growth cones growing on different CAM or ECM substrates is described. The results of these experiments suggest that substrate molecules may affect the cytoskeleton of growth cones, and this could potentially underlie the morphological changes observed with timelapse videomicroscopy. Together, these results provide important information about the effects that CAMs and ECM molecules have on individual growth cone motility and behavior. In the developing embryo, these effects may be imperative for correct pathfinding by individual growth cones.
LITERATURE CITED


CHAPTER 2

Neurite Growth on Different Substrates: Permissive versus Instructive

Influences and the Role of Adhesive Strength
INTRODUCTION:

Understanding how axons are guided to their targets is a central problem in developmental neurobiology. Several different mechanisms have been proposed to subserve this function. These include diffusible tropic molecules, channels or substrate molecules patterned to provide pathways to targets. Substrate bound adhesion molecules, such as cell adhesion molecules or extracellular matrix molecules could regulate axon growth through a variety of mechanisms. Letourneau performed an important experiment that indicated axons might choose pathways based on simple adhesive hierarchies (Letourneau 1975). He found that axons prefer to grow on polycrithine and collagen rather than palladium coated surfaces and that axons prefer palladium to tissue culture plastic; these preferences correlated with the strength of binding of the growth cones to the various surfaces. The positive correlation between adhesivity and growth cone selectivity established by these studies has been widely interpreted to indicate that axons in vivo choose between different paths by growing along the more adhesive one (Lockerbie 1987; Sanes 1989) for reviews).

Over the past few years several observations have been made that suggest that substrate molecules can have complex influences on neurite growth. For example, leech neurons in vitro show no selectivity for growth on two different substrates but the morphology of the neurites (Chiquet and Nicholls 1987) and the localization of Ca++ channels on the neurites (Ross. Arechiga et al. 1988) differ depending on the substrate. Similarly, neurites of sympathetic neurons in vitro express either axonal or dendritic properties depending on the substrate (Lein and Higgins 1989). These observations indicate that a substrate can have an instructive influence on neurite growth, causing dramatic alterations in plasma membrane and cytoskeletal composition. Neurites have been found not to show oriented growth when extending over a gradient of laminin
(McKenna and Raper 1988) and the rate of growth and neurite initiation is relatively insensitive to laminin concentration (Buettner and Pittman 1991). Such findings suggest that some substrate molecules may be permissive, inducing neurite elongation, but not instructive, in the sense of directing growth in a particular direction along a pathway or indicating which way to go at an intersection between two pathways. Substrates also may have an inhibitory influence on neurite outgrowth. Bonhoeffer and associates have shown that temporal retinal axons, when given a choice between anterior and posterior tectal membranes, prefer to grow on membranes from anterior tectum, and that this selectivity is due to the presence of an inhibitory substance on the posterior tectum (Walter, Kern-Veits et al. 1987). Similarly, proteoglycans (Snow, Lemmon et al. 1990) and proteins produced by oligodendrocytes (Schwab and Schnell 1991) have been shown to inhibit axon growth in ways that can direct axon pathway selection. Finally, dorsal root ganglion neurites prefer laminin to collagen yet their growth cones bind more weakly to laminin than collagen (Gundersen 1987) suggesting that a neurite's response to a substrate is independent of its adhesiveness.

In the studies reported here, we have examined three of the cell adhesion molecules and extracellular matrix molecules that promote neurite growth. We compared the ability of L1, N-cadherin, and laminin, as well as poly-L-lysine (PL) to promote neurite outgrowth, to affect the degree of neurite fasciculation, and to influence the choices neurites make between substrates with different adhesivities for growth cones. L1 and N-cadherin were chosen because of their presence on axons, their proven ability to support neurite growth and their likely importance in axon growth and fascicle formation in vivo. Laminin was tested because of its potent neurite growth promoting properties and its presence in developing axon pathways. We tested it in two presentations, either on nitrocellulose or PL because 1) nitrocellulose was used to adhere the two CAMs yet many other investigators use PL to increase laminin binding
to plastic and 2) preliminary experiments showed that neurites responded slightly differently to laminin with the two different methods. PL was used because it represents the interesting case of a substrate that supports neurite growth yet probably does this despite the absence of a bona fide cell surface receptor on neurites for this substrate. Moreover, PL is widely used as a substrate for neuronal attachment in cell culture experiments. If growth cone behavior is determined principally by degree of adhesion to a given substrate, then PL should be a good substrate for predicting growth cone behavior. To interpret the following experiments, it was crucial that the experiments be done on a relatively homogeneous population of growth cones. For this reason, the very long neurites that emerge from chick retinal explants were chosen. They grow from retinal ganglion cells and the neurites express axonal markers such as L1, F11, neurofascin and phosphorylated neurofilament.

We found that the adhesivity of a substrate was not correlated with the degree of neurite fasciculation or growth rates. A major and unanticipated finding was that neurites showed little selectivity when permitted to choose between L1, N-cadherin or laminin. Because L1 is much more adhesive than N-cadherin or laminin this suggests that relative substrate adhesiveness is not a principle factor in guiding axon growth.

MATERIALS AND METHODS:

Preparation Of Substrates:

Nitrocellulose was obtained from Schleicher and Schuell. Laminin was obtained from Gibco, Collaborative Research, or Upstate Biotechnologies. Poly-L-lysine (PL) was obtained from Sigma. Rat L1 and chick L1 were purified using an affinity column conjugated to 74-5H7 (Lemmon, Farr et al. 1989) or 8D9 (Lemmon and McLoon
1986) antibodies respectively. N-cadherin was purified using antibody NCD-2 (Hatta and Takeichi 1986) as described by Bixby and Zhang (Bixby and Zhang 1990).

Corning 35 mm tissue culture dishes were coated with a solution of nitrocellulose dissolved in methanol (Lagenaur and Lemmon 1987). Laminin dishes were coated with a 1mg/ml solution of laminin in Ca\(^{2+}/Mg\)^{2+} free Hank's buffer (CMF). Rat L1, chick L1 and N-cadherin were used, with protein concentrations of 0.8, 0.3, and 0.1 mg/ml respectively determined by the Pierce BCA Protein Assay. These concentrations of substrates were assumed to produce a saturating effect on growth cone behavior because 10 fold dilutions of the substrates resulted in identical responses from the neurites. Substrates were applied by spreading 20μl of substrate solution across a NC-coated dish, followed by blocking with 1 ml of fetal bovine serum (FBS) and storage in 1.5 ml plating medium. Plating medium consisted of Dulbecco's modified Eagle's medium (DMEM)/10% FBS/5% Chick Embryo Extract (CEE)/ and penicillin/streptomycin/fungizone (PSF). Dishes were incubated at 37°C in 5% CO₂, 95% air while retinal explants were prepared.

Poly-L-lysine dishes were prepared by incubating 35 mm tissue culture dishes in 0.1 mg/ml PL in sterile distilled water for 3-4 hours at 37°C in 5% CO₂, 95% air. These dishes were rinsed twice with CMF and incubated in plating medium until used for retinal explant plating. For laminin-PL dishes, PL coated dishes were incubated with a solution of 10 μg/ml laminin in CMF at 37°C in 5% CO₂, 95% air. The laminin solution was then replaced with plating medium and incubated during preparation of retinal explants.

*Preparation Of Retinal Explant Strips:*

Fertilized White Leghorn chicken eggs were incubated in a humidified, forced-air incubator at 39°C. Embryonic day 7 or 8 (E7 or E8, corresponding to Hamburger and
Hamilton stages 28-29) embryos were used for cultures. The procedure for retinal explant cultures has been previously described (Halfter, Newgreen et al. 1983; Drazba and Lemmon 1990). Retinal explant strips were incubated at 37°C in 5% CO₂, 95% air for 40-48 hours before use for growth rates or blasting experiments.

_Growth Rate Assessment:_

Neurite growth rates on the various substrates were assayed with time lapse video microscopy. Cultures were removed from the incubator and either additional prewarmed (37°C) plating medium (DMEM/10% FBS/5% CEE/PSF) was added or the medium was exchanged with prewarmed HEPES-DMEM/10% FBS/5% CEE/PSF (pH 7.1). Dishes were then coverslipped and placed in a heated stage ring of a Sensortek TS-4 temperature controller on a Leitz Diavert microscope. The ring maintained the culture medium temperature at 37°C. The microscope was in a Plexiglass incubation chamber to decrease temperature variability. A continuous flow of 5% CO₂, 95% air was pumped into the incubation chamber if the dish contained normal plating medium. Time lapse video images were obtained with a Javelin Chromachip II camera and Javelin Heliquad time-lapse video recorder at setting 216. Under these conditions cultures remained healthy for greater than 8 hours of recording time, although most recording sessions were limited to 8 hours maximum per dish. Neurite growth was reviewed on a NEC monitor, transparency traces made at approximately 30 minute intervals, and the distance measured between time points with a Bioquant Image Analysis System IV. A 10X objective was used for all measurements and growth rates were assessed as micrometers of growth per hour. Growth cones used for growth rate analysis were limited to single growth cones that grew unobstructed and without retraction during the videotaping.
Fasciculation Assessment by Scanning Electron Microscopy:

Explants with neurites were rinsed briefly in fresh medium cooled to 4\degree C. The explants were then fixed for 2 h at 4\degree C in 4% glutaraldehyde/4% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4. The fixed specimens were washed for 1 h at 24\degree C with 4% sucrose in 0.1 M sodium cacodylate, pH 7.4, then incubated for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer. A conductive coating was chemically deposited onto the specimen surface by two cycles of 15 min incubations in (a) 1% tannic acid buffered with 0.1 M sodium cacodylate, pH 7.4, followed by (b) 1% osmium tetroxide in 0.1 M sodium cacodylate (Postek, Howard et al. 1980). The specimens were dehydrated in an ascending ethanol series, transferred from 100% ethanol to Freon 113, and dried in a critical point drying apparatus. The dried coverslips were mounted onto aluminum stubs, coated with gold and viewed with a scanning electron microscope. Neurite fasciculation on each substrate was assessed visually and no obvious change in fasciculation was detected between 24 and 48 hour cultures on the same substrate. A quantitative assessment of fasciculation was made by photographing five random fields on each substrate at 800X. The widths of fascicles in each field were measured.

Growth Cone Blasting/Adhesivity Assay:

Adhesivity of retinal ganglion cell growth cones on various substrates was measured using a modification of an assay previously described (Gundersen and Barrett 1980). Explants in culture 40-48 hours were used to correspond to the growth rate data. Dishes were removed from incubation, the medium was exchanged with prewarmed (37\degree C) HEPES-DMEM/10% FBS/5% CEE/PSF (pH 7.1), and the dishes were placed in the same microscope system used to measure growth rates. Individual growth cones
were observed using a 20X objective, and a grid eyepiece was used to measure the distance from the leading edge of the growth cones and the pipette tip.

The blasting apparatus consisted of a 1 mm diameter capillary pipette pulled to a 1.5 \( \mu \text{m} \) opening, filled with plating medium, and attached to a General Valve Picospritzer II via tubing. The driving force of the Picospritzer was \( N_2 \) gas maintained at 40 pounds pressure. Once a single growth cone was sighted, the pipette tip was positioned with a micromanipulator 25 \( \mu \text{m} \) directly in front of the leading edge of the growth cone. The pipette was angled at 45° to the dish surface and lowered into the dish medium until the tip was within 5 \( \mu \text{m} \) of the dish surface. Medium was squirted through the pipette tip opening toward the growth cone at separate intervals beginning with a 10 millisecond duration and increasing by 10 milliseconds with each trial. This "blasting" process was continued until the growth cone was completely dislodged from its original position on the dish surface, or until a blast duration of 200 msec was reached. While the values obtained from these experiments cannot be related to any physical force such as mdyne (Heidemann, Lamoureux et al. 1990), subjective impressions of the experimenters were that a blast of 40 msec produced a relatively weak puff incapable of dislodging most cells or their processes and that a blast of 160 msec produced a gale capable of detaching large adherent aggregates of cells from the dish. At least 20 growth cones were blasted from each of the five substrates using the same pipette tip. Dishes used for blasting were kept out of the incubator for a maximum of 20 minutes. Only those experiments in which the same pipette was used for blasting of growth cones on all five substrates were compiled for the final adhesivity assessment, although incomplete experiments (<5 substrates with the same pipette) also showed the same adhesivity trend. Pipette tips were examined after each blasting experiment and the opening was measured.
Statistical Analysis:

The Statview II statistical analysis program was used to analyze data from the growth rate and growth cone blasting experiments. Analysis of variance was done on both data sets. However, because the growth cone blasting experiments did not produce parametric data (McCall 1970), pair-wise comparisons were made between different substrates using the Mann-Whitney U test.

Choice Experiments:

Alternating, parallel lanes of substrates were applied to tissue culture dishes with a silicone matrix generously donated by F. Bonhoeffer, using a modification of a previously described procedure (Vielmetter, Stolze et al. 1990). Briefly, the sterilized matrix was laid with the channel side facing the surface of a NC-coated Falcon 60 mm tissue culture dish. A 10μl aliquot of fluorescein labeled substrate was injected into the inlet channel, incubated for 3 minutes at room temperature, then replaced with a fresh substrate aliquot for five separate incubations of 15 total minutes. Fresh substrate exchanges were used to compensate for the small volume of the channels and to ensure even binding of the substrate to the dish. The substrate lanes were then saturated with inert protein by injecting a 20μl aliquot of sterile 4% Bovine Serum Albumin (BSA) in dH2O. Three separate injections of BSA at room temperature were used for a total incubation time of 10 minutes. The lanes were then washed with three quick passes of CMF and the matrix was removed from the dish. The second substrate was applied to the sites between the channel lanes by spreading 20μl of substrate across the entire lane area. The second substrate was incubated in the covered dish for 15 minutes at room temperature, with frequent tilting of the dish to ensure even coating. Dishes were then rinsed with CMF and stored in plating medium in 37°C in 5% CO2, 95% air until used for plating (up to 16 hours). For each experiment, substrate #1 was applied to the
channels in one dish with substrate #2 spread across the lane area, while substrate #2 was applied to the channels in a second dish with substrate #1 spread across the lane area. This provided a control that demonstrated that the order of substrate application did not affect neurite outgrowth preference. As with the conditions used in the other experiments described above, it was concluded that the substrate molecules were applied in high concentrations that would saturate the NC-coated plastic because 10 fold dilutions of the substrates produced similar neurite growth characteristics. Inclusion of fluorescent markers with the different adhesion molecules showed that there was no detectable cross-contamination of adjacent lanes. Retinal explants were laid perpendicular to the substrate lanes in these dishes, and the neurite outgrowth was assessed at 24, 48, and 60 hours after plating. At the completion of the experiment, the cultures were fixed with 1% paraformaldehyde, 0.01% glutaraldehyde in 0.1M phosphate buffer for 45 minutes, followed by two 0.1M phosphate buffer rinses. The cultures were coverslipped and the position of fluorescent substrate lanes confirmed by viewing with an epifluorescence Leitz microscope.

RESULTS:

Neurite growth rates:

Growing neurites were observed with timelapse video equipment and growth rates of the neurites were determined. Visual observation of explant cultures after 24 or 48 hours in vitro revealed that neurites grew rapidly and for long distances on laminin and relatively poorly on PL, while the other substrates had intermediate growth-promoting potentials. Measurements of growth rates confirmed these impressions of growth rates. The results shown in Table 1 indicate that neurite growth from retinal explants is slowest on PL followed in order by L1, N-cadherin, laminin-NC and laminin-PL.
Analysis of variance showed that the growth rates on the five substrates are significantly different at the 0.05 level.

**Fasciculation:**

Axons of projection neurons are usually found in bundles, growing in tracts in the CNS and in nerves in the PNS. The formation of these bundles is determined by choices the axons make; should they grow along other axons or should they grow through other terrains composed of extracellular matrix, glia, neuronal dendrites or somas? When they chose to grow along other axons they form bundles or fascicles. The degree of fasciculation of neurites is extremely difficult to quantify both *in vivo* and *in vitro*. To asses fasciculation we relied upon both qualitative and quantitative parameters: observation of growth cone encounters with neurite shafts during time lapse video, examination of fixed cultures using both light microscopic and scanning electron microscopic techniques and measuring fascicle width from SEM photographs. Time lapse videos indicate that fasciculation is primarily determined by growth cone behavior. When a motile growth cone encounters a neurite it may collapse and retract, it may adhere to the neurite and turn and grow along it forming a fascicle or it may cross under or over the neurite and continue to grow along the substrate. We never observed neurite shafts that were adherent at one point to zip up from the point of contact to some distant point to form a fascicle.

Comparison of patterns of neurite fasciculation on different substrates (see figure 1) revealed distinctive growth patterns for the different substrates. On poly-L-lysine, the neurites emerged slowly from the explant as large, highly interwoven mats of neurites. Bundles containing many neurites would exit the mats and grow relatively short distances on the PL. The time lapse videos revealed numerous lamellipodial and filopodial projections emerging from the growth cones and contacting either the PL or
adjacent neurites. In the vast majority of the cases the projections would fail to adhere to the PL but would usually form stable contacts with neuronal surfaces, and therefore form very compact fascicles. On laminin-NC the neurites emerge rapidly from the explants and tend to grow as multiple bundles, with each bundle containing many neurites although individual neurites were observed. The neurites on laminin-PL grew individually or as bundles with a few neurites. If a neurite growing on laminin encountered another neurite it would frequently alter its course to grow along the preexisting neurite. L1 and N-cadherin substrates produced the most defasciculated growth. Time lapse videos revealed that on L1 the neurites would emerge as a broad front of independent growth cones growing in close proximity to each other but always choosing to grow along the substrate and not on other neurites. When examining fixed cultures with the light microscope similar neurite growth patterns were observed on N-cadherin and L1. The scanning electron microscope, however, revealed obvious differences between neurites on L1 and N-cadherin; L1 produced a significantly more defasciculated pattern of growth. On L1 the neurites flatten out (Payne, Burden et al. 1992), resulting in a slightly larger mean fascicle diameter on L1 compared to N-cadherin. This is despite the fact that virtually all the neurites on L1 grow individually and not in fascicles while only about 20% of the neurites on N-cadherin were not in bundles with other neurites. The mean fascicle diameters on the various substrates were: L1, 0.884 microns ± 0.035; N-cadherin, 0.637 ± 0.036; laminin-PL, 1.384 ± 0.103; laminin-NC, 1.705 ± 0.121 (mean ± S.E.M.). N-cadherin fascicles were significantly different from fascicles on laminin at the 0.0001 level.

*Growth cone adhesion:*

Measurement of growth cone adhesion is difficult and may be influenced by factors such as growth cone size and shape, the number of receptors expressed on a growth
cone for a given substrate, and the number of adherent filopodia emerging from the
growth cone. While the adhesion data obtained in our experiments cannot be directly
related to any physical force, the data can be used to rank relative adhesion. The results
of five different experiments are presented in table 2. L1 was most adhesive followed
by poly-L-lysine, laminin-PL, N-cadherin and laminin-NC. The rankings obtained
from the experiments were very reproducible. In the five experiments there is only one
instance, in experiment 3, where there was a reversal in ranking between the two
substrates, N-cadherin and laminin-PL, that consistently had very similar adhesiveness.
Pair-wise comparisons of the substrates using the Mann-Whitney U test revealed that
different substrates had significantly different adhesiveness (at least at the .05 level) for
all possible combinations except N-cadherin versus laminin-PL. For this pair only one
of the 5 experiments met the 0.05 level criterion for being significantly different.
Therefore, we conclude that N-cadherin and laminin-PL have relatively similar
adhesiveness for chick retinal ganglion cell growth cones. In separate experiments the
adhesiveness of rat L1 and chick L1 were not significantly different from each other
(data not shown).

To determine if there is a correlation between growth cone adhesion to different
substrates and either fasciculation or neurite growth rate we chose to rank the results of
the different experiments because the measures of adhesion and fasciculation do not
meet the requirements of having magnitude, equal intervals and absolute zero point that
would permit direct correlation of the data (McCall 1970). If substrate adhesiveness
was positively correlated with neurite growth rate and defasciculation then a 3-
dimensional plot of this would appear like that in figure 2a. In our experiments, the
least adhesive substrate (laminin-NC) was given a rank of 1, the most adhesive
substrate (L1) was given a rank of 5 and the two substrates that were not significantly
different from each other (N-cadherin and laminin-PL) were given equal ranks of 2.5
(figure 2b). For comparison the mean growth rates on each substrate were used to rank the substrates. This analysis indicates there is a very poor correlation between growth cone adhesion and growth rates. Although there is a trend for the most adhesive substrates to have slower growth rates, neurites grew five times faster on the most adhesive substrate (L1) than on the 4th ranked substrate (PL). To examine the relationship between adhesion and fasciculation the substrate that produced the most defasciculated neurite growth (L1) was given a rank of 5 and the substrate that resulted in highly fasciculated growth (PL) was given a rank of 1. Again, there was a very poor correlation between adhesivity and degree of fasciculation. Neurites do grow in a highly defasciculated manner on L1, the most adhesive substrate, yet neurites are the most fasciculated on PL, the second most adhesive substrate. Also two substrates that were judged to be equally adhesive, N-cadherin and laminin on PL, gave very different patterns of fasciculation.

Choice experiments:

To determine the preferences of neurites for different substrates, we used a system that produces alternating lanes of different adhesion molecules (Vielmetter, Stolze et al. 1990). The advantage of this system is that after application of the first adhesion molecule, it is possible to block the nitrocellulose with a neutral protein before application of the second adhesion molecule to the alternate lanes. This greatly minimizes the amount of cross contamination between the parallel lanes of substrates. Inclusion of fluorescently labeled proteins with the adhesion molecules indicated no cross-contamination of the substrates, as assessed with a fluorescence microscope. When neurites were given a choice between laminin and poly-L-lysine, L1 and poly-lysine or N-cadherin and poly-L-lysine the neurites always grew on the glycoproteins and not on the poly-L-lysine (data not shown). Therefore, neurites can show substrate
preference in this assay. However, neurites showed relatively little selectivity when
given choices between laminin and N-cadherin, laminin and L1 or L1 and N-cadherin,
although there was a weak tendency for neurites to prefer N-cadherin (figure 3). In all
three cases neurites were observed to grow from one substrate onto the second then
continue to an adjacent lane of the first substrate. The order of substrate application did
not alter this result, consistent with a lack of cross contamination of adjacent lanes of
adhesion molecules. Due to the much more adhesive nature of L1, the failure of
neurites to localize on this substrate indicates that neurites are not selecting lanes by
preference for more adhesive substrates.

DISCUSSION:

The mechanisms that determine growth cone choice of appropriate pathways
through the developing central nervous system and embryo are undoubtedly complex
and diverse (Walter, Allsopp et al. 1990) (Reichardt and Tomaselli 1991) (Strittmatter
and Fishman 1991). There is general agreement that adhesion to a substrate is a
prerequisite for neurite growth and that filopodial or lamellipodial attachment to a
substrate may modulate the rate of neurite elongation (Lockerbie 1987; Bray and
Hollenbeck 1988). A reasonable prediction from these observations is that neurites
might grow more rapidly on more adhesive substrates. This is the case when artificial
substrates such as tissue culture plastic, poly-ornithine and poly-lysine are compared
(Letourneau 1975). In our experiments, when naturally occurring substrates for neurite
growth such as laminin, L1 and N-cadherin were compared there was, if anything, a
negative correlation between substrate adhesivity and neurite growth rate. This is more
consistent with recent computer modeling of cell migration which suggests that
migration rates show a biphasic response to substrate adhesiveness. Migration rates
increase as adhesiveness increases but eventually the migration rates decrease to zero on very adhesive substrates (DiMilla, Barbee et al. 1991). We observed, however, that the most adhesive substrate, L1, induced much higher neurite growth rates than the second most adhesive substrate, PL. One explanation for this observation is that cell surface receptors, such as L1 and integrins may interact with cytoplasmic second messenger systems (Bixby 1989; Schuch, Lohse et al. 1989; Tomaselli and Reichardt 1989) that actively regulate growth rate by controlling cytoskeletal assembly or disassembly or membrane insertion rates. Poly-lysine would not be expected to interact with similar signal transduction systems.

Neurite fasciculation in vitro and in vivo has been described as the result of competition between axon-growth cone and axon-axon adhesion versus growth cone-substrate adhesion. For example, if growth cone-substrate adhesion is relatively high then defasciculated neurites will be produced. Alternatively, if growth cone-axon adhesion is relatively high then highly fasciculated neurites will appear (Rutishauser, Acheson et al. 1988; Schubert and Klier 1991). Data from experiments in which antibodies were employed to disrupt fasciculation have been interpreted to support this notion. For example, antibodies to NCAM and L1 cause defasciculation of neurites growing on laminin. Moreover, the ability of antibodies to disrupt fasciculation suggests that fasciculation depends on decisions made at the growth cone. For example, addition of antibodies during neurite growth inhibits fasciculation (Rathjen, Wolff et al. 1987) yet the same antibodies added at the same antibody concentration to established fascicles of live neurites, in the "cartwheel assay," does not result in a defasciculation of the neurite cables (Chang, Rathjen et al. 1987). The results of the present experiments indicate that substrate adhesivity is a poor predictor of the degree of neurite fasciculation. Two substrates with relatively similar adhesivity, N-cadherin and laminin, produce very different patterns of fasciculation with fascicles on laminin.
being on average twice as thick as those on N-cadherin. Furthermore, growth cones growing on L1 and N-cadherin both produce highly defasciculated patterns of neurite growth, yet L1 and N-cadherin have extremely different adhesivities. Finally, poly-L-lysine, the second most adherent substrate, produces highly fasciculated outgrowth. Therefore, our findings suggest that the factors that cause neurite fasciculation are more complex than a simple competition between the adhesivity of growth cones for axons or the surrounding cells and matrix.

The ability of neurites to discriminate between different patterned substrates in vitro is well documented. One of the earliest examples of discrimination was the demonstration that neurites prefer poly-ornithine or collagen to palladium (Letourneau 1975). Based on such results Letourneau (Letourneau 1975) proposed that neurites choose between two different pathways in vivo based on their relative adhesiveness. This notion has been very appealing and investigators have attempted to infer the relative adhesiveness/affinity of growth cones for particular substrates in vivo based on growth cone morphology and pathway choice (Nardi 1983; Caudy and Bentley 1986). Gunderson, however, has reported that DRG neurites prefer laminin to collagen yet growth cones bind more weakly to laminin (Gundersen 1987) and have decreased areas of contact (Gundersen 1988) when compared to collagen and fibronectin. In analogous experiments examining olfactory neuronal migration, Calof and Lander have shown that migrating neurons prefer laminin to fibronectin yet bind less tightly to laminin (Calof and Lander 1991). These experiments indicate that when laminin and fibronectin are mixed, the laminin is anti-adhesive, resulting in decreased adhesion even in the presence of excess fibronectin, probably by altering the cell's ability to bind to fibronectin and not by steric hindrance. There have been a number of experiments over the past few years (reviewed by (Reichardt and Tomasselli 1991) that indicate ECM components that support neurite growth are permissive but do not provide instructive
information about direction of growth. Our results indicate this also may be true for N-cadherin and L1. Our experiments show that CNS neurites can cross between L1, N-cadherin and laminin, substrates with very different adhesivities for growth cones, going both from low adhesivity to high and vice versa. We also found that CNS neurites would not grow from N-cadherin or L1 onto a substrate with an intermediate degree of adhesivity, poly-L-lysine. These results indicate that neurites, when confronted with naturally occurring substrates, do not select particular pathways exclusively by relative adhesivity.

Our results should not be interpreted as indicating that CAMs and extracellular matrix molecules are unimportant in guiding axons to their targets. Rather, some adhesion molecules such as laminin, L1 and N-cadherin, which almost all axons can grow on, are likely to play an essential permissive role in providing pathways that promote rapid axon growth. Particular pathways might be selected by the expression of other receptors, such as TAG-1 (Dodd, Morton et al. 1988) or neurofascin (Rathjen, Wolff et al. 1987), on a subpopulation of axons that recognize a given pathway. CAMs and SAMs also might act to signal particular pathways to certain classes of neurons by initiating active responses within their growth cones (Strittmatter and Fishman 1991). Recently integrins and immunoglobulin superfamily adhesion molecules have been implicated in activating conventional second messenger systems that regulate intracellular kinases (Bixby 1989) (Danilov and Juliano 1989) and Ca++ (Schuch, Lohse et al. 1989). CAMs and SAMs are also thought to interact directly with the cytoskeleton (Horwitz, Duggan et al. 1986; Pollerberg, Schachner et al. 1986) (Nagafuchi and Takeichi 1988). In this way, growth cone adhesion to a substrate may be instructive and produce alterations in growth cone behavior in ways that direct growth along particular pathways. If this view is correct, then CAMs and SAMs
should be viewed as cell surface receptors capable of signal transduction besides being mediators of cell adhesion.
Figure 1. Scanning electron micrographs of patterns of fasciculation on different substrates. Photographs on the right show higher magnifications of fields indicated in boxes in the photographs to the left. A and B show the dense mat of highly fasciculated neurites that grow on poly-L-lysine. C and D illustrate neurite fasciculation on laminin on nitrocellulose. Bundles of fasciculated neurites are also evident on laminin on poly-L-lysine in E and F. On N-cadherin (G and H) neurites are much more defasciculated than on laminin but can still form small bundles of tightly packed neurites (indicated with arrows in H). On L1 (I and J), even in regions of very dense neurite growth, the neurites rarely form compact bundles of neurites. Scale bars: A, C, E, G, I = 50 mm. B, D, F, H, J = 10 mm.
Figure 1. Scanning electron micrographs of patterns of fasciculation on different substrates.
Figure 1. Scanning electron micrographs of patterns of fasciculation on different substrates.
Figure 2. Correlation between substrate adhesiveness, neurite growth rates and neurite fasciculation. 2a shows the expected graph if increasing adhesiveness produced faster neurite growth and less fasciculation. 2b shows the plot of the actual data obtained. See results for discussion.
Figure 3. Parallel lane choice experiments. 3a Neurites crossing alternating lanes of N-cadherin and laminin. 3b Neurites growing across alternating lanes of L1 and laminin. 3c Neurites growing over alternating lanes of N-cadherin and L1.
TABLE 1. Neurite growth rates on different substrates.

SEM = standard error of the mean

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<tr>
<th>RANK</th>
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<th>MAXIMUM GROWTH RATE OBSERVED (µm/HR)</th>
<th>MEAN GROWTH RATE (µm/HR)</th>
<th>SEM</th>
<th>NUMBER OF GROWTH CONES EXAMINED</th>
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TABLE 2. Mean blast duration in msec. required to dislodge growth cones. SEM = standard error of the mean

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CHAPTER 3

Growth Cones are Actively Influenced by
Substrate-bound Adhesion Molecules.
INTRODUCTION:

The formation of proper neural connections in the brain and with peripheral tissues occurs during early stages of development. In many cases, axons must traverse long distances through a complex environment comprised of a variety of molecules and cell types before reaching their target tissues. The development of proper connections is critical and may involve several different mechanisms. In some cases it appears that axons grow passively in preformed pathways surrounded by tissues that act as mechanical barriers (Krayanek and Goldberg 1981)(Oakley and Tosney 1991). In other cases, axonal growth is selective in that axons actively navigate to their targets by detecting positive and negative chemical cues in their environment that influence pathway choice (e.g. neurotrophins, cell adhesion molecules, extracellular matrix molecules, and proteoglycans)(Landmesser 1984; Silver and Rutishauser 1984; Lumsden and Davies 1986; Dodd and Jessell 1988; Heffner, Lumsden et al. 1990; Brittis, Canning et al. 1992; Tang, Landmesser et al. 1992).

Each axon possesses a highly motile, membranous structure at its terminus called a growth cone that acts as a sensory organ during this outgrowth period. In vitro, growth cones continually extend and retract broad membranous sheets of lamellipodia, and finer spikelike filopodial processes that can be up to several tens of microns long. Growth cones are thought to use these membranous extensions to actively explore their environment and interact with cells in their path.

Growth cone pathfinding has been studied extensively in several model systems. The retinotectal pathway has proven to be a good system for these studies because it is well-characterized and readily accessible (Holt and Harris 1993). During development of the chicken retinotectal pathway, retinal ganglion cells (RGCs) send out axons by embryonic day 2.5 (stage 16) toward the optic fissure (Lemmon, Burden et al. 1992).
The axons travel through the optic nerve to the chiasm where they cross to innervate the tectum on the contralateral side.

During this period of outgrowth, the axons come into contact with several different cell adhesion molecules (CAMs) and extracellular matrix (ECM) molecules including L1/8D9 (Lemmon and McLoon 1986), N-cadherin (Matsunaga, Hatta et al. 1988), R-cadherin (Inuzuka, Miyatani et al. 1991), Neural Cell Adhesion Molecule (NCAM) (Thiery, Duband et al. 1982; Schlosshauer, Schwarz et al. 1984) and laminin (Cohen, Burne et al. 1987). L1/8D9 and NCAM are members of the immunoglobulin superfamily of adhesion molecules, and have been shown to bind in a homophilic and heterophilic fashion (Lemmon, Farr et al. 1989);(Rutishauser, Hoffman et al. 1982; Cole and Akeson 1989; Sonderegger and Rathjen 1992; Brummendorf, Hubert et al. 1993). L1/8D9 is mainly confined to axons in the nervous system where it is thought to play an important role in axonal fasciculation (Lemmon and McLoon 1986), whereas NCAM is ubiquitously expressed in the developing embryo (Rutishauser 1983). N-cadherin and R-cadherin are members of the cadherin family of adhesion molecules that bind in a homophilic and heterophilic fashion (Takeichi 1990; Inuzuka, Miyatani et al. 1991). N-cadherin is expressed by neuronal and non-neuronal cells and may play a role in heterotypic cell-cell interactions (Drazba and Lemmon 1990; Letourneau, Shattuck et al. 1990; Letourneau, Roche et al. 1991) as well as in morphoregulatory events in the embryo (Hatta, Takagi et al. 1987). Laminin is an ECM molecule expressed in basement membranes that is recognized by integrin receptors (Hynes 1992). When purified from embryonic tissues and used as a substrate in culture, these CAMs and ECM molecules support the outgrowth of neurites from different types of neurons, as well as the adhesion and migration of several non-neuronal cell types (Reichardt and Tomaselli 1991; Doherty and Walsh 1992; Lemmon, Burden et al.
1992). Therefore, it is thought that these molecules, individually or in concert, may play similar roles in the embryo.

Several theories have been offered for how CAMs and ECM molecules might direct the outgrowth of axons toward their appropriate target tissues. Initially, it was suggested that axons choose the most adhesive pathway available (Letourneau 1975; Letourneau 1975; Hammarback, McCarthy et al. 1988), but in vitro experiments by Gundersen and Lemmon et al. (Gundersen, 1987; Gundersen, 1988; Lemmon, et al., 1992) have shown that the relative adhesiveness of a substrate is a poor predictor of the choices neurites make between two substrates.

Another theory is that the binding of CAMs and ECM molecules may be transduced into an intracellular signalling cascade that is important for growth cone pathfinding. It has been shown that the addition of purified CAMs or antibodies directed against the CAMs results in changes in intracellular pH, Ca^{+2}, IP_{2} and IP_{3} levels (Schuch, Lohse et al. 1989; Halbach, Taylor et al. 1992). Doherty and colleagues have provided evidence suggesting that a G protein-dependent activation of Ca^{+2} channels may be involved in neurite outgrowth on N-cadherin, L1 or NCAM-expressing 3T3 cells, but not on laminin (Doherty, Ashton et al. 1991; Williams, Doherty et al. 1992);(Safell, Walsh et al. 1992). An erbstatin-sensitive tyrosine kinase (Williams, Walsh et al. 1994), and consequently arachidonic acid (Williams, Walsh et al. 1994), also appear to be involved in the upstream activation of the calcium channels for neurite outgrowth. The application of FGF onto cerebellar neurons also results in activation of these intracellular messengers and enhanced neurite outgrowth (Williams, Furness et al. 1994). In addition, cerebellar neurons from src- mice display impaired neurite outgrowth on L1 but not laminin, suggesting a partial role for pp60^{c-src} in L1-mediated axonal growth (Ignelzi, Miller et al. 1994). Together, these results suggest that cellular
interaction with CAMs produces intracellular signals that are involved in the initiation of neurite outgrowth and possibly other, as yet undefined, cellular responses.

RGC growth cones and neurites in vitro display distinct morphologies and growth characteristics dependent upon the substrate (Lemmon, Burden et al. 1992; Payne, Burden et al. 1992). On L1/8D9, the growth cones are large, fan shaped and predominantly lamellipodial, although they exhibit quite a few short filopodia. Neurites on L1/8D9 form a dense but defasciculated monolayer. In contrast, growth cones on laminin display a smaller lamellipodial region and have fewer filopodia, but the filopodia are more than one third longer than those on L1/8D9. Neurites on laminin are highly fasciculated and tend to grow as tight bundles of several neurites together. On N-cadherin, growth cones are lamellipodial in nature with many short filopodia. These growth cones are smaller than those on L1/8D9, but are significantly larger and flatter than those on laminin. RGC neurites on N-cadherin are generally defasciculated, although small bundles of two to three neurites are observed occasionally. These differences in morphology are likely a result of changes in cytoskeletal architecture that result from the binding of CAM or ECM receptors with their appropriate ligand. Distinct growth cone responses to different CAMs and ECM molecules may signify CAM-derived signals used by the growth cone to make a behavioral change. It is important to understand how abrupt changes in the types of molecules encountered can affect growth cone behavior, since RGC growth cones grow through a rapidly changing cellular environment en route to their target.

In the studies reported here, timelapse videomicroscopy was used to examine chick RGC growth cones as they interacted with a sharp border between L1 and laminin, or N-cadherin and laminin. The results indicate that growth cones are actively affected by the substrate. For example, as a growth cone crossed over onto a new substrate, it underwent dramatic morphological changes to a shape characteristic for the new
substrate even though its neurite and the proximal portion of the growth cone remained on the initial substrate. A variety of behavioral responses were observed at border regions including cross without delay, collapse, retraction, and turning to remain on the initial substrate. These responses were associated with particular substrate border combinations, suggesting that contact with certain substrates may elicit signal cascades in the growth cone that are necessary for appropriate pathfinding.

MATERIALS AND METHODS:

Preparation of Substrates:

Black, round nitrocellulose filters from Sartorius (0.45μm pore size, 25 mm diameter) were used to secure retinal explants to the culture dish. Nitrocellulose (0.45μm pore size, grade BA85) for coating of coverslips was obtained from Schleicher and Schuell. Laminin was obtained from Gibco-BRL and polylysine from Sigma. Rat L1 and chick L1/8D9 were purified from brains using an affinity column conjugated with 74-5H7 (Lemmon, Farr et al. 1989) or 8D9 (Lemmon and McLoon 1986) antibodies respectively. N-cadherin was purified using antibody NCD-2 (Hatta and Takeichi 1986) as described by Bixby and Zhang (Bixby and Zhang 1990).

The culture chamber consisted of a 60 mm tissue culture dish (Falcon) with a hole drilled through the bottom. An acid-washed glass coverslip (Fisher) was glued to the bottom of the dish with Silastic medical adhesive (Dow Corning), allowed to dry, and the dish was UV irradiated. The coverslip was coated with 0.1 mg/ml polylysine in dH2OH overnight at 37°C, then rinsed with distilled water and air dried. A thin layer of nitrocellulose solution (Lagenaur and Lemmon 1987) was spread across the coverslip and allowed to dry. A 2x20 mm piece of sterile filter paper was laid on the coverslip and saturated with the first substrate protein (L1, laminin, or N-cadherin; 100μg/ml) for
a 15 minute incubation. A small amount of rhodamine-labeled BSA was included in this solution to mark the position of the substrate stripe. The stripe was then blocked with 2% bovine serum albumin (BSA) in Ca\(^{2+}\)-Mg\(^{2+}\)-free Hank’s buffer (CMF) for 10 minutes, the filter paper was removed and the stripe was rinsed with distilled water. The second substrate protein was spread adjacent to and in contact with the first substrate stripe, and incubated for 15 minutes. This procedure resulted in a continuous coating of substrate proteins, with an abrupt transition between the two substrates at a point denoted as the border region. The entire coverslip was then blocked with 2% BSA in CMF for 10 minutes and stored at 37°C covered with RPMI medium until used for plating (approximately 30 minutes).

**Preparation of retinal explant strips:**

Retinal explants from White Leghorn chick embryos (embryonic day 7, corresponding to Hamburger and Hamilton stages 29-30) were plated as previously described (Halfter, Newgreen et al. 1983; Drazba and Lemmon 1990) using media consisting of RPMI 1640/10% fetal bovine serum/2% chick serum (CS)/penicillin-streptomycin-fungizone (PSF). Retinas were cut at 350μm intervals, perpendicular to the optic fissure. Explants were inverted and oriented parallel to the substrate border as they were placed on the coverslips in culture dishes. This resulted in outgrowth of retinal ganglion cell (RGC) axons at a roughly perpendicular orientation to the border. The cultures were incubated overnight at 37°C in 5% CO2, 95% air before use for the timelapse studies.

**Timelapse Videomicroscopy:**

The cultures were removed from the incubator after approximately 24 hours, and the medium exchanged with prewarmed Hepes-DMEM/10% FBS/2% CS/PSF. The
cultures were coverslipped and transferred to the stage of the microscope (Zeiss Axiosver 405M) that was fitted with an incubation chamber and maintained at 37°C (Nikon NP-2 incubator). After a one hour equilibration on the microscope stage, time lapse recording was begun. For these experiments, a 40x or 100x oil immersion phase objective was used. Since the RGC growth cones were very sensitive to light, images were collected at low light levels with a silicon intensified target (SIT) camera (Dage MTI-65 or a Hamamatsu SIT camera with an Argus 10 processor). A Uniblitz shutter was used to minimize light exposure during intervals between image collection. Images were collected at thirty second intervals (Image-I, Universal Imaging), with 64 frames averaged per image, and stored on a Panasonic optical disk for later analysis. Cells remained healthy for longer than ten hours on the microscope stage, with individual growth cones recorded for as long as four hours with no adverse effects.

Since the RGC growth cones tended to grow out in a wave from the explants, individual growth cones at the front of the wave were generally selected for timelapse. These growth cones were usually 50-100μm away from the border region at the start of recording to allow for assessment of growth rate, and their path toward the border was clear of any other neurons or debris. An image of the fluorescent border boundary was collected at the start and finish of each recording sequence to ensure that the dish had not moved during recording, and to define specifically where the growth cone contacted the border region. No transition could be seen between the two substrates using phase contrast optics, suggesting that the border did not present a physical barrier.

Analysis of Timelapse Sequences:

Growth cones that contacted the border region without interference from other growth cones were selected for further analysis. Several types of interactions were observed, including an immediate cross onto the other substrate, collapse or retraction
upon contact with the border, turning and growing along the edge of the first substrate, or a delay of variable time followed by a cross or turn. Growth cones displayed any combination of these interactions, and were therefore examined individually. These interactions were analyzed by preparing traces of each growth cone on transparencies at 1-15 minute intervals as the growth cone grew on the first substrate, interacted with the border and grew beyond. The traces were used to categorize growth cone response at the border, measure changes in growth rate, and illustrate the morphological changes in the growth cones as they grew onto a new substrate. Growth rate and growth cone area measurements were made from the traces with a Bioquant Image Analysis System IV.

The timelapse series shown in figures 1-6 were produced as follows. Using the Image-1 software, a dashed line was painted along the fluorescent border image for each field. This line was overlaid onto the corresponding phase contrast images, and the selected regions from each image were pasted together in a timelapse series image. Each resultant image was enhanced further (Photoshop 2.0.1 software) by changing the background to a lighter level. Timelapse series images were printed using a Kodak XLS 800 digital printer.

**RESULTS:**

Timelapse videomicroscopy was used to examine growth cones as they interacted with a sharp border region between L1/8D9 or N-cadherin and laminin. The use of retinal explants for these experiments provided a homogeneous group of ganglion cell axons that grew out in a wave toward the border region. Due to this outgrowth pattern, those growth cones at the front of the wave were used for analysis. It should be noted that growth cones behind the front of the wave also behaved similarly even though they
were in contact with other neurites and growth cones at the time of border contact. At the border region, several behaviors were observed such as crossing onto the second substrate, turning to remain on the first substrate, collapse or transient retraction. These behaviors were observed individually (e.g. immediate cross) or in combinations (e.g. collapse followed by a turn) and were characteristic of the order in which substrates were contacted. Delays of variable length were also observed after contact with the border region.

*Growth cone interactions at the interface of laminin and N-cadherin:*

RGC neurites growing on laminin tended to remain in fascicles of various sizes. For the timelapse studies described below, small diameter fascicles were followed as they grew toward the border region. When growth cones growing on laminin contacted the N-cadherin border, 100% of the growth cones (n=22) crossed onto N-cadherin (Table 1). During the cross, the growth cones underwent a characteristic shape change from a small, bulbous morphology on laminin to a much larger and flattened appearance as they migrated onto the N-cadherin (Figure 1). This change occurred progressively: The portion of the growth cone on N-cadherin displayed the flattened morphology even though the rear portion of the growth cone and the neurite were in contact with laminin and displayed morphology appropriate to laminin. The growth cones were also observed to separate from one another as they progressed onto the N-cadherin, resulting in defasciculation of the neurites. One growth cone turned at the border and grew for 15 minutes on the laminin before finally crossing. Only 14% (n=3) of the growth cones delayed after contact with the border for an average time of 17 minutes before crossing onto the N-cadherin (see Table 1). Interestingly, 41% (n=9) were observed to collapse transiently upon contact with the N-cadherin, but none retracted a distance greater than ten microns prior to crossing. The collapse occurred
coincident with or within five minutes of border contact, and lasted less than five
minutes on average before the growth cone recovered to its initial size. Collapse
resulted in a slight decrease in growth rate in three cases that was recovered within the
next five minutes. In the remaining six cases, collapse had no effect on growth rate.

In the opposite situation, 30 growth cones were analyzed as they initiated growth on
N-cadherin and then interacted with a laminin border. Since neurites on N-cadherin
tended to grow out in a wave from the explants, only the growth cones at the wave
front were used for the analysis. Of these, 60% (n=18) crossed onto the laminin.
Three of these growth cones delayed an average of 40 minutes at the laminin border
prior to crossing (Table 1). It should be pointed out that the growth cones at the wave
front and those arriving later were observed to congregate at the border region before
crossing onto the laminin (not shown). In many cases, individual growth cones
extended long, thickened filopodia-like processes onto the laminin several times before
crossing (Figure 2). Most growth cones crossed as bundles of two or more at a time,
and growth cones behind the wave front tended to cross onto laminin by fasciculating
along neurites of growth cones that had crossed previously. One growth cone crossed
onto laminin and grew for a brief period, then turned back to grow on the N-cadherin
for the remainder of the recording session. This growth cone was included in both the
cross and turn categories in Table 1. Overall, 43% (n=13) of the growth cones turned
at the laminin border. None of these growth cones were observed to later cross onto
laminin, but rather, grew on N-cadherin at the border for the remainder of the recording
session (Figure 3). Seven of the growth cones illustrated an average delay prior to
turning of 24 minutes after contact with the laminin border. In the group of growth
cones at the wave front, 30% (n=9) collapsed transiently, and 10% (n=3) retracted
briefly by 12 microns on average upon contact with the border. Collapse occurred
coincident with or within five minutes of border contact. In seven cases, recovery
occurred within five minutes of collapse, even though retraction was associated with two of these cases. In the remaining two cases of collapse, recovery occurred within 10-50 minutes. Collapse resulted in a transient (less than 5 minutes) decrease in growth rate in five cases, but had no effect on growth rate in the remaining three cases. Five growth cones turned to grow along the border after the collapse response, whereas the remainder crossed onto the laminin. Comparison of the frequency of behaviors observed in this substrate combination with those of growth cones initiating on laminin (see Table 1) suggests that the order of substrate contact significantly affects growth cone response.

The growth cone morphology changes observed at border regions occurred rapidly after initial contact (Figure 7). Growth cones initiating on N-cadherin underwent a 40% decrease in area within the first minute after contact with the laminin border. This change occurred even though the majority of growth cones had contact only via leading edge lamellipodium. The area changed little during the next few minutes, but decreased by a total of 57% after completed cross onto laminin. In growth cones initiating on laminin, the area increased by 20% within the first minute of contact with the N-cadherin border, but increased by an additional 70% within the following four minutes. After the cross onto N-cadherin was completed, the area increased by 188% over the initial area observed on laminin. Representative traces of growth cones undergoing morphological changes at N-cadherin-laminin borders are shown in Figure 8. These results indicate that the growth cones are very sensitive to changes in substrate, and actively respond to new substrate cues through modifications in morphology.

*Growth cone interactions at the interface of laminin and L1/8D9:*

Growth cones that initiated growth on laminin were analyzed as they contacted a border of L1/8D9 (n=17). All of these growth cones crossed onto the L1/8D9, with
only 35% (n=6) delaying an average of 7 minutes prior to crossing (Table 1). As growth cones crossed onto L1/8D9, they progressively changed from the small, bulbous morphology characteristic of laminin to a much larger, fan-shaped growth cone (Figures 4, 5). Growth cone advance onto the L1/8D9 resulted in a progressive defasciculation of neurites similar to that observed on N-cadherin. None of the growth cones were observed to turn at the border to continue growth on laminin. Interestingly, 59% (n=10) of the growth cones collapsed upon contact with the border, while only 18% (n=3) retracted at least 10 μm (average 10μm). Collapse occurred less than five minutes after contact, and all growth cones recovered within five minutes after collapse. In four cases, collapse was associated with a transient decrease in growth rate, but in the remaining six cases no effect on growth rate was observed. All growth cones that retracted displayed a collapse just prior to retraction. A few growth cones were observed to collapse after border contact, and upon second contact the growth cone changed morphology to one appropriate to L1/8D9 even though most of the growth cone was still on laminin (Figure 4).

In the opposite situation, growth cones initiating growth on L1/8D9 (n=23) crossed onto laminin less frequently (75% of the time, n=17) (Fig. 6), and 35% (n=6) of them delayed 44 minutes on average prior to crossing (Table 1). RGC growth cones on L1 tend to grow out as a wave of neurites from the retinal explants. Therefore, growth cones at the front of the wave were selected for analysis although those behind the wave front illustrated similar behaviors. The growth cones were observed to congregate at the border region in a similar fashion as on N-cadherin, and usually crossed onto laminin as bundles of two or more growth cones. Often, the growth cones produced filopodial or lamellipodial extensions that appeared to sample the laminin several times prior to crossing. Growth cones behind the wave front tended to cross onto laminin by fasciculating along the neurites that had crossed previously. 35%
(n=8) of the growth cones turned at the border to continue growth on L1/8D9. Four of these growth cones turned without delay, while the remaining four delayed an average of 40 minutes prior to turning. Two of the growth cones that turned at the border later crossed onto the laminin, but the remaining six growth cones that turned maintained growth on the L1/8D9 at the border for the remainder of the recording session. A small percentage of growth cones (17%, n=4) collapsed upon first contact with laminin, but none of them were observed to retract greater than 10 microns. In all cases, collapse occurred coincident with or within eight minutes of contact, and each growth cone recovered within five minutes of collapse. Two of the growth cones that turned to grow along the border displayed a collapse prior to turning. Collapse was associated with a slight decrease in growth rate that lasted for less than five minutes in three cases, and a more extensive decrease in growth rate that lasted for approximately twenty minutes in one case. Comparison of the frequency of behaviors in this substrate combination suggests that the order of substrate contact significantly affects growth cone response.

Growth cone area measurements made prior to, during, and after border contact revealed that growth cones responded rapidly to a change in substrate (Figure 7). For example, growth cones initiating on L1/8D9 showed a nearly 40% decrease in area within the first minute of contact with the laminin border. Growth cones completing the cross onto laminin displayed a 62% total decrease in area from original levels observed on L1/8D9. In the opposite situation, growth cones initiating on laminin showed a 48% increase in area within the first minute of contact with the L1/8D9 border. After the cross onto L1/8D9 was completed, the area increased by 169% over the initial area observed on laminin. The rapid change in area cannot be attributed to differences in adhesivity alone since the region of contact with the new substrate was confined to the leading edge lamellipodium in the majority of growth cones measured.
Representative examples of the extent of changes in growth cone area are shown in Figure 8. Together, these results show that CAMs and ECM molecules used as a substrate can actively influence growth cone morphology and behavior.

**DISCUSSION:**

Timelapse videomicroscopy is a powerful tool that provides important information about cell behavior. It has been used by many investigators to examine a variety of cell-cell and cell-substrate interactions in vitro, as well as in vivo processes such as growth cone pathfinding (Chien, Rosenthal et al. 1993; O'Connor and Bentley 1993). In this report, timelapse videomicroscopy was used to examine RGC growth cones as they interacted with a sharp border between two substrates normally encountered in vivo. Growth cones displayed active responses upon contact with a new substrate: Crossing, turning to remain on the first substrate, collapse and retraction were responses that occurred with frequencies dependent upon the order of substrate contact. Extensive growth cone morphology changes were also observed during interaction with a new substrate. The variety of behaviors exhibited at borders suggests that the growth cones were actively influenced by contact with a new substrate.

Growth cones of different neuronal origin have been shown to exhibit a number of behaviors in response to contact with other cells or molecules (Kapfhammer, Grunewald et al. 1986; Kapfhammer and Raper 1987; Honig and Burden 1993; Oakley and Tosney 1993). Some of these responses could be considered passive or a response to an attractive cue, for example, crossing behavior. In all substrate combinations examined, growth cones were observed to cross onto the new substrate with fairly high frequency. This response was not surprising since RGC axons grow readily on these substrates when presented individually. An important finding was that growth cones
illustrated a weak preference for N-cadherin or L1/8D9 over laminin. This slight preference was exhibited in two ways. First, more than one third of the growth cones initiating on L1/8D9 or N-cadherin turned after contact with the laminin border to remain on the first substrate. Second, a significant delay in forward growth was illustrated by greater than one third of the growth cones after contact with the laminin border, regardless of whether the growth cones crossed or turned. Together, these results suggest that laminin was not inhibitory to the growth cones, but rather was the less preferable substrate of the two available. This result was not detected in previous studies of cultures at fixed timepoints, in which a population of neurites was examined after interaction with several consecutive borders between substrates (Lemmon, Burden et al. 1992). In those studies, the response of the overall neurite population was examined instead of individual growth cones, therefore the indicators of weak preference discussed above were not apparent. In the present study, all growth cones initiating on laminin crossed onto either L1/8D9 or N-cadherin with only a short delay after contact. These results further support the idea that although the substrates tested were permissive for growth, they elicited different responses from the growth cones.

Growth cones growing from L1/8D9 or N-cadherin to the laminin border often extended several filopodial processes transiently onto the laminin, as if sampling the new substrate. This behavior was observed regardless of whether the growth cone eventually crossed onto the laminin or turned at the border. Filopodia have been shown to act as individual sensory units (Davenport, Dou et al. 1993) required for appropriate pathfinding (Bentley and Toroian-Raymond 1986; Chien, Rosenthal et al. 1993), and a stabilized filopodial contact is capable of changing growth cone directionality in vivo (O'Connor and Bentley 1993). Therefore, the filopodia may have been acting as sensors to integrate the substrate cues available prior to execution of the final observed behavior.
Growth cones underwent dramatic changes in morphology upon contact with the border region and during growth on the new substrate. Growth cones initiating on L1/8D9 or N-cadherin underwent a 40% decrease in area within the first minute of lead edge contact with laminin. This rapid decrease in size occurred prior to contact by the entire growth cone with the laminin, and may be explained by activation of some sort of intracellular signal (Hynes 1992) which spread throughout the growth cone from the site of laminin contact. A laminin-induced change in growth cone morphology has also been observed by Rivas and colleagues using soluble laminin puffed onto sympathetic neurons (Rivas, Burmeister et al. 1992). Therefore, laminin appears to induce somewhat universal morphological changes in growth cones of different neuronal origin. After growth cones completed crossing onto the laminin, the growth cone area was observed to decrease a total of approximately 60%, a value which corresponds well with previous measurements (Payne, Burden et al. 1992). In the converse situation, growth cones initiating on laminin underwent a 20% increase in area within the first minute of N-cadherin contact, and an additional 70% increase during the following four minutes. A completed crossing onto N-cadherin resulted in a 188% total increase in growth cone area. Growth cones contacting L1/8D9 from laminin underwent an approximately 50% increase in size within the first minute that changed little during the following four minutes. Once the growth cones had completed crossing onto L1/8D9, the growth cone area was observed to increase a total of 169%. These results demonstrate an enlargement of growth cone area induced by CAM-contact when growth cones initiate on laminin. Again, since the growth cones changed size quickly and were not in full contact with the CAM when the change occurred, this change is probably elicited via activation of an intracellular signal. Several investigators have suggested that neurite growth stimulated by N-cadherin or L1/8D9 is dependent upon the activation of second messenger pathways (Doherty and Walsh 1992; Halbach,
Taylor et al. 1992; Doherty and Walsh 1994; Ignelzi, Miller et al. 1994). It is possible that specific changes in growth cone morphology and motility in response to contact with CAMs may also be a result of activation of second messenger pathways, but this has not yet been shown. The change in growth cone area is not primarily due to adhesive properties of the new substrate, since RGC growth cones adhere equally to laminin and N-cadherin (Lemmon, Burden et al. 1992), but are significantly larger on N-cadherin than on laminin (Payne, Burden et al. 1992).

A collapse response has often been attributed to an inhibitory interaction, especially when associated with retraction of the growth cone (Kapfhammer and Raper 1987; Ivins and Pittman 1989; Davies, Cook et al. 1990) (Davies and Cook 1991). Retraction often occurs in conjunction with a significant delay, for example, Kapfhammer and Raper described delays of greater than 15-30 minutes after the retraction of a retinal growth cone from a sympathetic neurite, and vice versa (Kapfhammer and Raper 1987). During the course of the retraction that they observed, the growth cone maintained residual filopodial contacts with the neurite, and reapproached the same neurite after the delay. These results are somewhat different than those described here. First, only one third of the collapse responses observed were associated with a retraction, and in all cases the growth cones recovered to their initial size and resumed forward movement within five minutes of retraction. None of the growth cones maintained filopodial contact with the border region during retraction. Of the growth cones initiating on N-cadherin that retracted at the laminin border, two turned at the border and one crossed onto laminin. All of the growth cones initiating on laminin crossed onto L1/8D9 after recovery from retraction. Due to the transient nature of the retraction, it seems that this response was not indicative of an inhibitory interaction. Rather, the initial collapse/retraction may signify a large, transient change in a second messenger that is produced as a result of contact with the new substrate. If
adaptation to the second messenger occurs within the growth cone, then a second contact with the new substrate might not be expected to elicit a retraction, as was observed here.

Two thirds of the growth cones that collapsed did so without an accompanying retraction. Collapse always coincided with contact with the border, or within a few minutes, suggesting that interaction with the new substrate evoked this response. Of growth cones initiating on L1 or N-cadherin, greater than one half were observed to turn at the border following collapse. Collapse was most common in growth cones initiating on laminin, and in this case was always followed by crossing onto the adjacent CAM. These results suggest that collapse may be a general behavior that occurs in response to abrupt changes in the growth environment, and may not be indicative of inhibitory influences in all cases. The substrate molecules used in this study have been suggested to interact with the cytoskeleton as a result of ligand-receptor binding (Hirano, Nose et al. 1987; Hynes and Lander 1992; Davis, McLaughlin et al. 1993). In addition, the distribution of RGC growth cone cytoskeletal elements differs depending upon the growth substrate (see Chapter 4). Therefore, the collapse observed here may be due to specific rearrangements of cytoskeletal elements triggered by contact with a new growth-promoting molecule. Rearrangements in growth cone cytoskeletal elements, specifically filamentous actin and microtubules, in response to collapse have been demonstrated by other investigators (Lankford and Letourneau 1989; Fan, Mansfield et al. 1993).

Contact with stimulatory growth molecules, such as L1/8D9, N-cadherin and laminin, may result in collapse that is a consequence of activation of a second messenger pathway. One of the more likely candidates involved in this response is intracellular calcium ([Ca$$^{++}$$]). In some (Cohan, Connor et al. 1987; Bandtlow, Schmidt et al. 1993), but not all (Ivins, Raper et al. 1991), cases of growth cone
collapse, changes of $[\text{Ca}^{++}]_i$ within the growth cone have been observed. It is interesting that the triggering of L1 or NCAM on the surface of PC12 cells, cerebellar neurons or dorsal root ganglion neurons by purified CAMs or CAM-directed antibodies has been reported to alter $[\text{Ca}^{++}]_i$ (Schuch, Lohse et al. 1989; Halbach, Taylor et al. 1992). In addition, neurite outgrowth stimulated by L1, N-cadherin or NCAM appears to require calcium influx through N- and L-type calcium channels (reviewed in Doherty and Walsh 1994). Local increases in $[\text{Ca}^{++}]_i$, up to a threshold level, appear to be necessary for new veil formation and growth (Cohan, Connor et al. 1987; Goldberg 1988). Above this threshold level, increased $[\text{Ca}^{++}]_i$ results in collapse of growth cones (Cohan, Connor et al. 1987) and inhibits neurite elongation (Silver, Lamb et al. 1989). It is possible that several signalling pathways may lead to the same behavioral change in the growth cone, namely collapse. This idea is supported by results of Igarashi and colleagues, in which growth cone collapse induced by several different stimuli was shown to be mediated by G protein-coupled receptors (Igarashi, Strittmatter et al. 1993).

Due to the many instances of growth cone collapse observed in the present study when growth cones encountered growth-promoting rather than growth-inhibiting substrates, one must reassess the function of the collapse response. Does collapse with its associated disassembly of the existing cytoskeleton promote efficient reorganization of the cytoskeleton into a new configuration more suitable for a novel environment? For example, would collapse speed the transition from an integrin-based mode of translocation to a CAM-mediated one? The different time course observed in the present study for collapse and delay induced by growth-promoting substrates compared with inhibitory molecules such as those observed by Kapfhammer and Raper (Kapfhammer and Raper 1987; Kapfhammer and Raper 1987) raises the possibility that they are produced by not just quantitative differences in intracellular signals such as
calcium levels, but may imply that there are qualitative differences such as the involvement of different second messenger systems. In support of this idea, Finnegan and colleagues determined that collapse and retraction of goldfish retinal ganglion cell axons in response to monoclonal antibody 8A2 was due to elevated phosphorylation levels mediated by protein kinase C and a tyrosine kinase (Finnegan, Lemmon et al. 1993). Further studies on the proximal events in CAM and integrin mediated signals in growth cones are clearly required in order to understand the function of these molecules in axonal pathfinding.
Figure 1. Timelapse series of a growth cone initiating growth on laminin and contacting N-cadherin at a border region. Images of the growth cone are shown at 0:00 (a), 4:00 (b), 5:00 (c), 5:30 (d), 9:30 (e), and 14:30 (f) minutes progressed time from first frame (a) (time denoted as minutes:seconds). The border between substrates is indicated by the dashed line. The selected field area was moved to the left in b, e, and f to accommodate for forward progress of the growth cone. Compare the small, bulbous growth cone on laminin (a) with the much larger, flattened growth cone on N-cadherin (e, f). Note the rapid change in morphology of the growth cone that occurs progressively (b-d), and the defasciculation of neurites (e, f) after contact with the N-cadherin border. Calibration bar=10 μm.
Figure 2. Timelapse series of a growth cone initiating growth on N-cadherin and contacting laminin at a border region. Images of the growth cone are shown at 0:00 (a), 8:00 (b), 10:00 (c), 14:00 (d), 15:00 (e), 42:30 (f), 50:30 (g), and 59:00 (h) minutes progressed time from first frame (a) (time denoted as minutes:seconds). The border is indicated by the dashed line. Note the collapse of the growth cone after first contact with laminin (c), and the long delay from initial contact until completed cross (b-h, 51:00 minutes). Thick filopodial processes were extended onto the laminin (d) and retracted multiple times (not shown) prior to final cross (h), as if the growth cone was sampling the new environment. Calibration bar=10 μm.
Figure 3. Timelapse series of a growth cone initiating growth on N-cadherin and contacting laminin at a border region. Images of the growth cone are shown at 0:00 (a), 14:30 (b), 19:30 (c), 30:00 (d) 33:00 (e), and 46:00 (f) minutes progressed time from first frame (a) (time denoted as minutes:seconds). The border is indicated by the dashed line. The growth cone approaches the border at a nearly perpendicular angle, but after contact with laminin (b, c), it turns to continue growing on N-cadherin (f). Note the thick processes extended onto the laminin (d) for sampling of the substrate even though the main portion of the growth cone remains on N-cadherin. The selected field area was moved up in f to accommodate for forward progress of the growth cone. Growth cones just out of the field above crossed onto the laminin substrate. Calibration bar=20 μm.
Figure 4. Timelapse series of a growth cone initiating growth on laminin and contacting L1/8D9 at a border region. Images of the growth cone are shown at 0:00 (a), 7:30 (b), 9:30 (c), 16:30 (d), 21:30 (e), and 37:30 (f) minutes progressed time from first frame (a) (time denoted as minutes:seconds). The border is indicated by the dashed line. The growth cone collapses (c) within minutes of initial contact with the L1/8D9 (b). Upon recovery and second contact with the L1/8D9 (d), the growth cone is observed to rapidly change to the large, fan-shaped morphology normally observed on L1/8D9 even though a large portion of the growth cone remains on laminin. The growth cone crosses rapidly onto the L1/8D9 after second contact with the border (d-f). Calibration bar=10 μm.
Figure 5. Timelapse series of a growth cone initiating growth on laminin and contacting L1/8D9 at a border region. Images of the growth cone are shown at 0:00 (a), 8:30 (b), 11:00 (c), 13:30 (d), 17:00 (e) and 28:00 (f) minutes progressed time from first frame (a) (time denoted as minutes:seconds). The border is indicated by the dashed line. Note that the growth cone collapses within minutes of initial contact with L1/8D9 (c), but recovers rapidly for continued forward progress (d). Minimal delay is observed between initial contact and resultant cross onto the L1/8D9 (b-e). Compare the small, bulbous growth cone on laminin (a) with the large, fan-shaped growth cone on L1/8D9 (f). Calibration bar=20 μm.
Figure 6. Timelapse series of a growth cone initiating growth on L1/8D9 and contacting laminin at a border region. Images of the growth cone are shown at 0:00 (a), 6:00 (b), 8:30 (c), 10:30 (d), 13:30 (e), and 26:30 (f) minutes progressed time from first frame (a) (time denoted as minutes:seconds). The border is indicated by the dashed line. Compare the large, fan-shaped morphology of the growth cone on L1/8D9 (a, b) with the smaller, bulbous growth cone on laminin (f). The growth cone decreases size rapidly within minutes of initial contact with laminin (b-d), and in this case does not delay long before crossing onto the laminin. Calibration bar=20 μm.
Figure 7. Comparison of mean growth cone area. The growth cone area was measured during growth on the first substrate prior to border contact, one minute after contact (C+1 min), five minutes after contact (C+5 min) and after completed cross onto the second substrate. (a) Growth cones initiating on laminin and crossing onto N-cadherin (n=11). (b) Growth cones initiating on N-cadherin and crossing onto laminin (n=5). (c) Growth cones initiating on laminin and crossing onto L1/8D9 (n=11). (d) Growth cones initiating on L1/8D9 and crossing onto laminin (n=5). Mean results from each category were compared using the paired student’s t-test; * p<0.05, + p<0.01, ** p<0.005, ***p<0.0005, +++ p<0.0001.
Figure 8. Summary of morphologic changes during border interaction.
Representative traces of growth cones prior to border contact, one minute after border contact, five minutes after border contact, and after completed cross onto the second substrate.
Table I. Growth cone interactions at substrate borders*

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<th>Collapse</th>
<th>Retract (&gt;10 μm)</th>
<th>Delay</th>
<th>Ave. Delay (minutes)</th>
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<tr>
<td>Laminin -&gt; N-cadherin</td>
<td>100</td>
<td>4</td>
<td>41</td>
<td>0</td>
<td>14</td>
<td>17 (cross) 0 (turn)</td>
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<tr>
<td>N-cadherin -&gt; Laminin</td>
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<td>43</td>
<td>30</td>
<td>10</td>
<td>33</td>
<td>40 (cross) 24 (turn)</td>
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*Results listed as percent of total.


CHAPTER 4

L1/8D9, N-cadherin and Laminin Induce Distinct Distribution

Patterns of Cytoskeletal Elements in Growth Cones
INTRODUCTION:

Growth cones are the specialized sensory structures present at the terminus of growing neuronal processes. Being highly motile organelles, growth cones undergo a variety of shape changes in culture due to extension and retraction of filopodial and lamellipodial processes. These terminal processes are thought to interact with cues in the environment to produce directed growth toward the appropriate target in vivo (Bentley and Toroian-Raymond 1986; O'Connor, Duerr et al. 1990; Lin and Forscher 1993). In neurons, cytoskeletal proteins provide the framework for the overall shape of growth cones, neurites and cell bodies. The cytoplasmic domains of growth cones can be divided into two distinct regions based on organelle content, thickness and motility (Bridgman 1992). The peripheral region of the growth cone consists of the lamellipodial and filopodial processes, which are thin, highly dynamic extensions of membrane that are seemingly devoid of membranous organelles. The most abundant cytoplasmic element in this region is actin, present in both globular and filamentous forms. The central region of the growth cone is the portion adjacent to the neurite which consists predominantly of microtubules (MTs), neurofilaments (NFs) and membranous organelles. This region has greater cytoplasmic thickness, and represents the distal-most site of neurite assembly. Disruption of the actin cytoskeleton results in the protrusion of MTs from the central region into the periphery of the growth cone, suggesting that the high density of actin normally present in the peripheral region excludes MTs from occupying this site (Forscher and Smith 1988).

Changes in growth cone motility appear to be due to alterations in the cytoskeleton network, namely in actin filaments and MTs (Mitchison and Kirschner 1988; Smith 1988; O'Connor and Bentley 1993; Bentley and O'Connor 1994). Polymerization of globular actin into filaments allows rapid extension of filopodia from the growth cone,
as well as formation of transient lamellipodial veils (Smith 1988; Lewis and Bridgman 1992; Sheetz, Wayne et al. 1992). Distinct populations of actin have been observed within growth cones, supporting the idea that one population may be involved in protrusion of peripheral processes whereas the other may be stationary and necessary for substrate anchorage (Lewis and Bridgman 1992). Experiments using cytochalasins to disrupt actin filaments have shown that the underlying actin cytoskeleton within growth cones is necessary for directed growth in vitro (Marsh and Letourneau 1984) and correct pathfinding in vivo (Bentley and Tordoian-Raymond 1986; Chien, Rosenthal et al. 1993). Also, several actin binding proteins have been localized to growth cones by immunocytochemical methods and may play a role in the polymerization and stabilization of actin microfilaments (Letourneau and Shattuck 1989; Sobue and Kanda 1989). Previous experiments have indicated a possible role for calcium in the regulation of actin filament assembly (Lankford and Letourneau 1989), and several actin binding proteins are regulated by calcium dependent processes (Forscher 1989). Therefore, fluctuations of intracellular calcium concentration via second messenger systems may modify the actin cytoskeleton.

Alterations in growth cone shape upon target contact appear to be initiated by rearrangements in microfilaments, and become stabilized due to recruitment of MTs for the final result of vectorial growth (Lin and Forscher 1993). MTs are also necessary for fast axonal transport of molecules from the cell body to the growth cone and for axonal elongation (Martenson, Stone et al. 1993). Disruption of the mechanisms controlling MT assembly results in blockade of neurite advancement and compromises growth cone motility (Letourneau and Ressler 1984; Bamburg, Bray et al. 1986). Within the growth cone, MT assembly is a dynamic process that must be precisely controlled to allow for both motility and consolidation of MTs into neuritic bundles. Tubulin undergoes several post-translational modifications including phosphorylation,
acetylation and deetyrosination that may play a partial role in MT assembly and stabilization (Serrano and Avila 1990). Other candidates for the control of MT bundling and interactions with actin filaments include microtubule associated proteins (MAPs): MAPs occupy neuronal processes and growth cones, and are closely associated with MTs (Gordon-Weeks 1991). One MAP, MAP 1B, is post-translationally phosphorylated by a casein-kinase II-like activity, with phosphorylation induced during neurite outgrowth (Diaz-Nido, Serrano et al. 1988). Phosphorylated MAP 1B is distributed in a gradient which is highest near the growth cone, and lowest near the cell body (Mansfield, Diaz-Nido et al. 1991). Together, these results suggest that MAP 1B may play a role in conjunction with MTs in neurite elongation processes that occur in the growth cone.

The third main constituent of the neuronal cytoskeleton is neurofilament (NF), a type IV intermediate filament triplet consisting of three proteins called NF-L, NF-M, and NF-H (Liem 1993). NFs are necessary for stabilization of neurites, yet their involvement in growth cone motility is less clear. Initially, it was thought that polymerized NFs were maintained as a static group with little exchange of subunits. More recently, experiments using fluorescence-energy transfer of a labeled NF-L protein in vitro (Angelides, Smith et al. 1989), and fluorescence photobleaching of labeled NF in vivo (Okabe, Miyasaka et al. 1993) have suggested that subunit exchange does occur between soluble NF proteins and assembled NFs. Interestingly, studies by Lee and colleagues (Lee, Carden et al. 1987) have shown that NF-M subunits are highly phosphorylated in axons, but are non-phosphorylated in cell bodies. A similar result was provided by Sternberger and colleagues using antibodies against NF-H subunit (Sternberger and Sternberger 1983). These results provide evidence for the existence of multiple isoforms of NF subunits, although distinct functions based on phosphorylation level remain to be shown.
As outlined above, the growth cone motility machinery consists of a complex group of protein-protein interactions that may be differentially regulated by changes in intracellular calcium levels and phosphorylation. As yet, it is unclear what specific intracellular events provide linkage between extracellular stimuli and cytoskeletal changes. On the surface of growth cones are receptors for a variety of cell adhesion molecules (CAMs) and extracellular matrix (ECM) molecules that are normally encountered in vivo. Interaction with certain CAMs and/or ECM molecules may provide guidance information, either by delineation of permissive pathways for growth (Gundersen 1987; Lemmon, Burden et al. 1992) or through an intracellular signalling cascade that results from receptor binding (Doherty and Walsh 1992; Doherty and Walsh 1994). This type of signal could be conveyed to the adjacent cytoskeleton to result in rearrangements of microfilaments, microtubules and ultimately in changes in growth cone motility.

Laminin is an ECM molecule present in basement membranes that is an adhesive, growth-promoting substrate for non-neuronal and neuronal cells in culture (Edgar 1991; Reichardt and Tomaselli 1991). The interaction of the integrin receptor for laminin with cytoskeletal elements has been well-documented (Hynes 1992; Gumbiner 1993), and appears to play a role in the formation of focal adhesions in migrating fibroblasts. A subset of focal adhesion proteins have also been localized to growth cones by immunocytochemical methods (Letourneau and Shattuck 1989; Sobue and Kanda 1989), and therefore may provide a similar bridge between integrin receptors and microfilaments. N-cadherin, a member of the calcium-dependent cadherin family of adhesion molecules (Takeichi 1988), is a potent neurite outgrowth-promoting substrate in culture (Bixby and Zhang 1990). The cytoplasmic domain of cadherins interacts with catenin proteins that may bridge an association with the actin cytoskeleton (Hirano, Nose et al. 1987; Gumbiner 1993) and therefore may also influence
cytoskeletal rearrangements. L1/8D9, a member of the immunoglobulin superfamily of adhesion molecules, is confined mainly to axons and growth cones and promotes extensive neurite outgrowth when used as a substrate in culture (Lemmon and McLoon 1986; Lagenaur and Lemmon 1987). Recent studies by Davis and colleagues have shown that the cytoplasmic domain of a L1/8D9-like glycoprotein binds with the cytosolic protein ankyrin, and in turn spectrin, for interaction with the cytoskeleton (Bennett 1990; Davis, McLaughlin et al. 1993). The binding of CAMs or ECM molecules with their receptors may trigger an intracellular response mediated by receptor interaction with specific cytosolic proteins. These interactions could result in a signalling cascade that is conveyed to the cytoskeleton for directional growth and stabilization of adhesion. The role of CAMs in the regulation of cytoskeletal changes has not been directly addressed, and may be a significant factor in alteration of growth cone motility and behavior.

In the previous chapter, timelapse videomicroscopy was used to show that retinal ganglion cell (RGC) growth cones actively undergo distinctive morphological changes in response to changes in the growth substrate. In this study, the cytoskeletal elements of growth cones growing on L1, N-cadherin or laminin, or across a sharp border between these substrates, were examined. The three main constituents of the neuronal cytoskeleton, the microfilaments, MTs. and NFs, were distributed in distinct patterns depending on which growth substrate was encountered by the growth cone. These results suggest that the growth substrate may actively influence cytoskeletal structure, resulting in morphological changes of growth cones.
MATERIALS AND METHODS:

Preparation of substrates:

Nitrocellulose was obtained from Schleicher and Schuell (0.45μm pore size, Grade BA85). Laminin was obtained from Gibco-BRL and polylysine from Sigma. Rat L1 and chick L1/8D9 were purified from brains using an affinity column conjugated with 74-SH7 (Lemmon, Farr et al. 1989) or 8D9 (Lemmon and McLoon 1986) antibodies respectively. N-cadherin was purified using antibody NCD-2 (Hatta and Takeichi 1986) as described by Bixby and Zhang (Bixby and Zhang 1990).

Acid-washed glass coverslips (Fisher) were incubated in 0.1 mg/ml polylysine in distilled water overnight at 37°C, then rinsed with distilled water and air dried. A thin layer of nitrocellulose solution (Lagena and Lemmon 1987) was spread across the coverslips and allowed to dry. To prepare alternating lanes of substrate, 1x20 mm pieces of sterile filter paper were saturated with the first substrate protein (L1/8D9, laminin, or N-cadherin; 100μg/ml) and laid on the coverslip approximately 1 mm apart for a 15 minute incubation. A small amount of rhodamine-labeled BSA was included in the substrate solution to mark the boundary of the substrate lanes. The lanes were then blocked with 2% bovine serum albumin (BSA) in Ca⁺²-Mg⁺²-free Hank’s buffer (CMF) for 10 minutes. The filter papers were removed, and the substrate solution was aspirated and allowed to dry briefly. The second substrate protein was spread across the lanes and incubated for 15 minutes. This resulted in a continuous coating of substrate protein, with abrupt transitions between the two substrates at points denoted as border regions. The entire coverslip was then blocked with 2% BSA in CMF for 10 minutes and stored at 37°C covered with RPMI medium until used for plating (approximately 30 minutes).
In some instances, retinal explants were plated on coverslips coated with only one substrate protein. In these cases, the substrate protein was spread across an entire nitrocellulose-coated coverslip and incubated 15 minutes at room temperature prior to blocking with 2% BSA in CMF.

**Plating procedure:**

Retinal explants from White Leghorn chick embryos (embryonic day 7, corresponding to Hamburger and Hamilton stages 29-30) were plated as previously described (Halfter, Newgreen et al. 1983; Drazba and Lemmon 1990) using media consisting of RPMI/10% fetal bovine serum (FBS)/2% chick serum (CS)/penicillin-streptomycin-fungizone (PSF). Retinas were cut at 350μm intervals, perpendicular to the optic fissure and the explants were inverted onto the coverslip in a parallel position with respect to the substrate border. This resulted in outgrowth of retinal ganglion cell (RGC) axons at a roughly perpendicular orientation to the border. The cultures were incubated overnight at 37°C in 5% CO₂, 95% air before fixing for immunocytochemistry (20-28 hours total growth time).

**Immunocytochemistry:**

Monoclonal antibodies against phosphoryrosine, pp60^src or pS9^fyn were obtained from UBI. YL1/2 monoclonal antibody against tubulin was obtained from Seralabs. Rhodamine phallloidin was obtained from Molecular Probes. The 5E10 monoclonal antibody against phosphorylated neurofilament (Landmesser and Swain 1992) was kindly provided by Dr. Lynn Landmesser (Dept. of Neurosciences, Case Western Reserve University).

Retinal explants prepared as described above were fixed at 20-28 hours after plating with ice-cold 4% paraformaldehyde, 0.01% glutaraldehyde in PEM buffer (80mM
Pipes, 5mM EGTA, 1mM MgCl₂, 3% sucrose) for 30-60 minutes, and rinsed with PEM buffer. The fixation protocol consisted of a gentle flow of fixative over the cells as media was removed from the dish, and resulted in no obvious change in growth cone morphology. The cells were permeabilized with 0.02% Triton in PBS (PBST) for 30 minutes, followed by a 30 minute incubation in 20% goat serum in PBST (GS-PBST). This protocol resulted in a gentle but complete permeabilization of growth cones. Primary antibodies were diluted with GS-PBST and incubated with the cells for 1.5 hours at room temperature, or overnight at 4°C. The explants were rinsed with PBS, and incubated with fluorescein-conjugated secondary antibody diluted in GS-PBST for 30-60 minutes. The explants were rinsed with PBS and coverslipped with mounting medium (0.5M Tris, 90% glycerol, 0.1% p-phenylenediamine, pH 9.0).

For staining of actin filaments, rhodamine phalloidin was used at a final concentration of 3.3x10⁻⁸ M in PBS. Cells were fixed and permeablized as described above, then incubated with rhodamine phalloidin for 30 minutes at room temperature, rinsed with PBS and coverslipped. Cells were photographed using Kodak T-MAX 400 film.

Quantitation of actin distribution:

Retinal explants were plated on L1, N-cadherin and laminin, and stained with rhodamine phalloidin as described above. Fifty growth cones were randomly selected on each substrate, and were visually tallied for actin distribution localized at the leading edge or spread throughout the growth cone. The position of the growth cone was also noted regarding contact with other growth cones or neurites. The results from four separate experiments were compared using the Anova test and unpaired t-test.
RESULTS:

Growth cones require a functional cytoskeleton for maintenance of lamellipodial veils and filopodia, motility, and also for directional growth (Letourneau 1983; Marsh and Letourneau 1984; Bray 1987; Cypher and Letourneau 1992; Lin and Forscher 1993). Retinal ganglion cell (RGC) growth cones exhibit characteristic morphologies dependent upon the substrate on which they are grown (Payne, Burden et al. 1992) see Chapter 3). In this study, immunocytochemical methods were used to examine the distribution of several cytoskeletal elements, kinases and phosphorylated tyrosine residues in growth cones growing on L1, N-cadherin or laminin individually, as well as on dishes coated with alternating lanes of these substrates.

Filamentous actin distribution varies depending upon the substrate:

Rhodamine-conjugated phalloidin was used to specifically label the filamentous actin (f-actin) within the RGC neurons. Growth cones displayed distinct f-actin patterns depending upon the substrate on which they were grown (Figure 1). For example, growth cones on laminin contained thick actin filaments that originated in the filopodia and extended deep into the growth cone central region. Other filaments of variable size filled both the peripheral and central regions of the growth cone, resulting in a dense network of actin filaments present throughout the growth cone (Figure 1B). This pattern is in sharp contrast with that observed in growth cones growing on L1. In the majority of these growth cones, f-actin bundles originating in filopodia extended into the peripheral region, but usually did not penetrate the central region of the growth cone. These bundles were accompanied with a dense meshwork of fine microfilaments that filled the peripheral region but also was not usually observed in the central growth cone body (Figure 1A). Growth cones on N-cadherin displayed two different f-actin
distribution patterns with equal frequency. The first pattern was similar to that observed on L1 in which the growth cone peripheral region was filled with a fine f-actin meshwork as well as thick bundles originating from the filopodia (Figure 1C). The second pattern consisted of f-actin bundles that filled the entire growth cone in a manner similar to that observed on laminin, except that in this case, an underlying fine meshwork of f-actin was also observed (Figure 1D). All neurites, regardless of substrate, displayed a typical cortical actin meshwork along their length. Fine, filopodia-like processes originating from the neurite were filled with parallel f-actin filaments that labeled more brightly than the cortical meshwork of the neurite.

To quantify the frequency of the observed f-actin patterns, fifty random growth cones growing on each substrate were examined in four separate experiments (Figure 2). Nearly 100% of the growth cones on laminin exhibited f-actin throughout the growth cone. In contrast, only 25% of growth cones on L1 exhibited this distribution pattern, while 75% displayed a much higher concentration of f-actin confined to the peripheral region. The distribution patterns on N-cadherin described above occurred with equal frequency. Comparison between growth cones growing alone or in contact with other growth cones or neurites did not reveal contact-dependent changes in f-actin patterns on any of the substrates tested (data not shown).

*Lamellipodial contact with a new substrate results in redistribution of actin filaments:*

F-actin is necessary for formation of lamellipodial and filopodial processes that underly directed growth cone movement (Marsh and Letourneau 1984; Bentley and Toroian-Raymond 1986; Chien, Rosenthal et al. 1993; Bentley and O'Connor 1994). The distinct morphological changes illustrated by growth cones interacting with substrate-bound molecules suggest active rearrangement of cytoskeletal elements (see Chapter 3). Therefore, the distribution pattern of f-actin was examined in growth cones
as they interacted with a sharp border between two substrates (Figures 3, 4). Growth cones initiating on laminin maintained thick f-actin bundles throughout the peripheral and central regions during filopodial or lead edge interaction with the L1 or N-cadherin border (Figure 3C). As growth cones progressed further onto L1 or N-cadherin, such that an extensive lamellipodial region was in contact with the new substrate, the f-actin redistributed mainly to the peripheral region where it formed a dense meshwork of fine filaments (Figures 3D, 4D). Only a few cases of growth cones progressing onto N-cadherin resulted in little change in f-actin distribution.

Growth cones initiating on L1 or N-cadherin often congregated at the border region prior to crossing, and crossed onto laminin in bundles of two or more. Occasionally, individual growth cones crossed onto laminin, and these were observed to have thick microfilaments throughout the peripheral and central regions only once they had extensive lamellipodial contact with the laminin (Figures 3B, 4B, 4C). A smaller percentage of growth cones turned upon contact with the laminin border to continue growth upon L1 or N-cadherin. In these growth cones, the central region remained on the L1 or N-cadherin, but a portion of the peripheral region at the side of the growth cone was often in contact with laminin. Under these conditions, growth cones on L1 contained f-actin that was mainly distributed in a meshwork that filled the peripheral region (Figure 3A). Growth cones on N-cadherin exhibited f-actin confined to the peripheral region or present as thicker bundles throughout the growth cone with equal frequency. The cortical actin staining was unchanged in neurites that crossed the border of any substrate combination.

*Characteristic microtubule distribution patterns are observed in growth cones on each substrate:*
The YL 1/2 antibody (Seralabs) against tubulin was used to examine MT distribution in RGC neurons. MTs present in neurites on all substrates appeared as a continuous tight bundle along the entire length of the neurite. At the intersection between the neurite and the base of each growth cone, the MT bundle loosened somewhat and separated into finer bundles (Figure 5). These bundles were fairly straight and generally remained confined to the central region of growth cones on laminin, although they were loosely associated (Figure 5C). Rarely, MTs penetrated the peripheral growth cone region to enter the base of filopodia that had formed thickened, stable contacts with adjacent neurites (not shown). In growth cones on L1, the MTs splayed out as more individual, gently curving filaments that penetrated into the periphery of the growth cone (Figure 5A). Many of the MTs reached near the leading edge of the growth cone, but only a few were observed to enter the base of filopodia. In growth cones on N-cadherin, the MTs were splayed similar to those in growth cones on L1, but were observed to reach the leading edge only in the largest growth cones (Figure 5B).

Alteration of microtubule distribution occurs after extensive lamellipodial contact with the new substrate:

The MT distribution was examined in growth cones as they interacted with borders between substrates (Figures 6, 7). Growth cones initiating on laminin displayed straight, compact bundles of MTs confined to the central region if contact with L1 or N-cadherin was limited to filopodial or lead edge contact (Figures 6A, B). The MTs were often observed to reorient as a group toward the site of CAM contact (Figure 6A). MTs began to loosen somewhat as the growth cone progressed further onto the new substrate, and were splayed throughout the central and into the peripheral region in growth cones that were greater than halfway across the border (Figures 6C, 7C).
In growth cones initiating on L1 or N-cadherin, MTs remained loosely splayed when the growth cone contacted laminin via filopodia or the leading edge. Interestingly, the MTs that penetrated the region in contact with the laminin in these growth cones became more closely associated with one another at that site (Figure 7A). Further growth cone contact with laminin resulted in thicker MT bundles that were slightly splayed or closely associated, but rarely penetrated the peripheral region of the growth cone (not shown). Once the growth cones were greater than halfway across the border so that their central region had extensive contact with laminin, the MTs were straighter and more tightly bundled (Figure 7B). A number of growth cones were observed to turn at the laminin border to continue growing on the L1 or N-cadherin. In these, only a small lamellipodial region remained in contact with the laminin, and the MTs maintained the splayed appearance normally observed on L1 or N-cadherin (Figure 7A). In addition, some MTs were observed to reorient toward the site of laminin contact and were more closely associated with one another in this region (Figure 7A).

*Growth cone neurofilament distribution varies with the substrate:*

Neurofilaments (NFs) are known to be involved in the stabilization of neurites and axonal growth (Lee and Cleveland 1994), but their role in growth cone structure and motility is less clear. The monoclonal antibody 5E10 (Landmesser and Swain 1992) was used to examine the distribution of NFs in growth cones. On all substrates, NFs were observed in a tight bundle that ran the length of the neurites, with bright patches flanked by dimmer regions. This pattern did not correlate with any obvious stimulus such as contact with other cells. Occasionally along the neurite, NFs were observed to spread apart into a loose ball that was usually localized to branch points or regions where filopodia originated from the neurite. In growth cones on laminin, the NF
bundles entered the base of the growth cone from the neurite, but remained relatively tightly bundled and did not progress far into the growth cone central region (Figure 8C). NFs in growth cones on L1 protruded much further into the growth cone central region, separated into more individual filaments, and splayed apart (Figure 8A). The NFs remained relatively straight and were rarely observed to protrude very far into the peripheral region of the growth cone. In growth cones on N-cadherin, the NFs penetrated the central region, loosened and splayed out as individual curved filaments that filled the growth cone body (Figure 8B). If two growth cones growing on either L1 or N-cadherin were in contact with one another, the NFs tended to redirect toward the contacting growth cone (Figure 8D).

_Neurofilament distribution changes upon extensive growth cone contact with a new substrate:_

The NF distribution was compared in growth cones that interacted with borders between two substrates. Growth cones initiating on laminin maintained tightly bundled NFs at the base of the growth cone when filopodia or the leading edge contacted L1 or N-cadherin (Figures 9C, 10D). NFs were first observed to enter the central region as a compact ball if a portion of the central region had crossed onto the CAM (not shown). Only after the majority of the growth cone had crossed onto the new substrate were the NFs observed to loosen somewhat and penetrate deeper into the growth cone body (Figure 10C). NFs in growth cones at borders were not observed to splay out as normally occurs on L1 or N-cadherin. This type of splaying was only observed after a completed cross.

Growth cones originating on N-cadherin or L1 maintained splayed NFs after lead edge contact with the laminin border. Yet, in cases where the central region of the growth cone contacted laminin, the NFs were observed to spread out along the border,
avoiding those regions of the growth cone that had crossed onto the laminin (Figures 10A, B). With further advancement onto laminin, NFs became bundled and did not progress as far into the central region of the growth cone (Figures 9A, B). Once the majority of the growth cone had crossed onto laminin, the NFs were observed to form a fairly tight bundle that was confined to the base of the growth cone. A few growth cones were observed to turn at the laminin border to continue growth on the L1 or N-cadherin. In these, a small region of lamellipodia maintained contact with the laminin, but the NFs remained splayed in the central region of the growth cone (Figure 9D).

*The distribution of phosphotyrosine, pp60src and p59fyn is unaffected by the growth substrate:*

pp60src and p59fyn are two members of the protein tyrosine kinase family. Both have been localized to growth cone preparations (Bixby and Jhabvala 1993) and may play a possible role in some aspect of growth cone motility and neurite outgrowth (Maness 1992). The distribution of these molecules and of phosphorylated tyrosine residues were examined in RGC growth cones growing on laminin, L1 and N-cadherin (Figure 11). On each of these substrates, the distribution pattern of all three antigens was similar. Staining was observed throughout all growth cone regions including filopodia, and also along the neurites. Staining was punctate in appearance, and was brightest in the central region of the growth cone, most likely due to the increased volume. Staining intensity for each antibody was similar on all substrates. This result was interesting since pp60src has been indicated to play a partial role in neurite outgrowth on L1 but not on laminin (Ignelzi, Miller et al. 1994).

In growth cones at borders between the substrates, the staining pattern was unchanged regardless of the amount of contact the growth cone made with the new substrate (Figure 12). This result illustrates that the two kinases, pp60src and p59fyn,
do not redistribute in response to the new substrate, yet it does not distinguish between the location of active versus inactive forms of these kinases. Since the phosphotyrosine staining pattern remained unchanged at border regions, it is unclear whether the phosphorylation of tyrosine residues plays a role in the morphological changes observed in growth cones at substrate border regions.

DISCUSSION:

These results show that the distribution pattern of microfilaments, MTs and NFs in growth cones is distinctive depending upon the growth-promoting molecule used as the substrate. Although the cytoskeletal elements remained essentially concentrated in specific regions, alterations in pattern were observed such as splaying out of more individual filaments, thicker bundles of filaments, and the presence of two distinct populations of actin filaments within the same growth cone (e.g. observed in growth cones on N-cadherin). Since RGC growth cones display distinctive morphologies and motility patterns depending upon the substrate (Payne, Burden et al. 1992), it is likely that substrate-receptor binding influences the cytoskeleton. This idea is supported by the observed alterations in cytoskeletal protein distribution in growth cones as they crossed borders between two substrates.

Growth cones on L1/8D9 and N-cadherin tend to be large, flat and lamellipodial in morphology (Payne, Burden et al. 1992). The large lamellipodial expanse of these growth cones may be supported by the observed randomly-oriented meshwork of f-actin. In addition to the f-actin meshwork, growth cones on N-cadherin often possessed thicker actin bundles that were oriented toward the filopodia. Both forms of actin distribution have been observed by others in lamellipodial regions of growth cones: The meshwork has been hypothesized to act as a scaffolding, the long radially
oriented fibers may provide substrate anchorage, and both populations may play a role in leading edge expansion (Lewis and Bridgman 1992). In comparison, growth cones on laminin are more bulbous in appearance, smaller in surface area, and are typified by longer filopodial processes (Payne, Burden et al. 1992). The actin filaments within these growth cones comprise bundles that originate in the filopodia and extend deep into the central region of the growth cone. Parallel-oriented actin bundles within filopodia have been observed by several investigators in growth cones of different origin, and have been equated with the force-generating properties of filopodia (Bray 1987; Lamoureux, Buxbaum et al. 1989). The bundles observed in growth cones on laminin are similar in appearance to stress fibers of non-neuronal cells that associate in regions of focal contacts with the integrin receptor (Hynes 1992).

The remaining cytoskeletal elements examined, MTs and NFs, were also distributed in distinct patterns within growth cones growing on different substrates. The lack of NF entry into growth cones on laminin is in sharp contrast with NF distribution throughout the central region of growth cones on either L1/8D9 or N-cadherin. This pattern may indicate that the site of neurite assembly differs in these growth cones: Assembly may occur at the base of the growth cone on laminin, but could occur more distally in growth cones on L1/8D9 or N-cadherin. A more likely explanation is that NFs were actively excluded by f-actin, since each cytoskeletal population was localized to distinct regions within the growth cones regardless of growth substrate. MTs were most concentrated in the central region of growth cones on any substrate. Often, they were observed to penetrate the peripheral region of growth cones on L1/8D9 or N-cadherin, and in some cases reached the base of filopodia. Similar results have been observed by other investigators in both static and timelapse studies (Bridgman 1992; Gordon-Weeks 1993; Lin and Forscher 1993), and have led to the conclusion that MTs comprise a highly dynamic population that is "captured" by actin filaments for directed
growth (Gordon-Weeks 1991; Gordon-Weeks 1993). As yet, it is unclear how actin and MTs interact with one another during this process, but several candidate proteins including actin binding proteins could play a role in this process. The MTs in growth cones on laminin were tightly associated, and remained confined to the central region almost exclusively. It is interesting that MTs were straight in growth cones on laminin, but assumed a more curved appearance in growth cones on both L1/8D9 and N-cadherin. Experiments in which taxol was used to stabilize MTs resulted in curved and looped MTs within growth cones (Letourneau and Ressler 1984). Therefore, MTs in growth cones on L1/8D9 and N-cadherin may be in a more stable state than those in growth cones on laminin. This stability may be necessary to maintain the large, lamellipodial expanse associated with these growth cones.

It is difficult to make firm conclusions about changes in growth cone cytoskeletal elements resulting from contact with substrate borders. In previous timelapse videomicroscopy studies (see chapter 3), some growth cones were observed to progress from one substrate to another without any delay, while many others collapsed upon contact with the new substrate or delayed for various periods of time prior to crossing onto the new substrate. The growth cones examined in this study were not followed with timelapse methods prior to fixation. Therefore, the recent history of these growth cones is unknown, for example, the amount of time they spent at the border or if they collapsed upon first contact with the new substrate. Nonetheless, some observations can be made about the cytoskeletal organization following border contacts. Actin was observed to redistribute within growth cones only after extensive leading edge contact with the new substrate. Morphology changes are likely a direct result of redistribution of the available actin subunits, since the rapid polymerization capability of globular actin is thought to account for motility of filopodia and lamellipodia (Gordon-Weeks 1990). Therefore, the observed progressive
redistribution of actin may indicate the first step in morphology change. The maintenance or stabilization of new morphologies may be due to recruitment of MTs into newly expanded regions (Gordon-Weeks 1993; Lin and Forscher 1993). This idea is supported by the finding that MTs were observed to reorient to the site of CAM contact, suggesting that they were somehow recruited, possibly by actin redistribution. The splaying of MTs that was observed in growth cones crossing onto L1/8D9 or N-cadherin from laminin is most likely due to a combination of two processes. A large pool of tubulin is present in growth cones and may have been utilized for polymerization of new MTs. Alternatively, the MT bundles may have been released from one another due to CAM influences on MAPs, resulting in splaying of the existing MT population. In concert with this idea, the MTs in growth cones crossing onto laminin from CAMs were observed to become bundled, which could be a result of reassociation of MAPs with the MTs. Of interest, NFs responded to local effects of substrate change, since they spread along the border in growth cones that contacted laminin from either L1/8D9 or N-cadherin. This could also be a direct response to actin redistribution, since thick actin bundles associated with an integrin mediated binding mechanism could potentially exclude the NFs from certain regions of the growth cone.

Rearrangement of cytoskeletal proteins may be regulated by post-translational modifications such as phosphorylation. NF-L (Sihag and Nixon 1990), NF-M (Lee, Carden et al. 1987; Sihag and Nixon 1990; Xu, Liu et al. 1992) and NF-H (Sternberger and Sternberger 1983; Clark and Lee 1991) have been shown to exist as phosphorylated forms in multiple classes of neurons. The amino terminal phosphorylation sites are targeted by protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA). Phosphorylation of these sites on NF-L results in disassociation of NF bundles and prevents reassembly in vitro (Nixon and Sihag 1991). Tubulin is post-translationally modified via phosphorylation, acetylation and
detyrosination, resulting in effects on MT assembly and stabilization (Serrano and Avila 1990). It is of interest that the substrate molecules used in this study have been shown to require protein kinases for various functions. For example, L1 and N-cadherin, but not laminin, utilize an erbstatin-sensitive protein kinase for promotion of neurite outgrowth (Williams, Walsh et al. 1994). The protein tyrosine kinase (PTK), \( pp60^{src} \), has also been implicated in the L1-mediated neurite outgrowth pathway (Ignelzi, Miller et al. 1994). In addition, the triggering of L1 on the surface of growth cone particles inhibits the \( pp60^{src} \)-dependent phosphorylation of tyrosyl residues on a subset of tubulin (Atashi, Klinz et al. 1992). The promotion of neurite outgrowth by laminin appears to require PKC (Bixby 1989; Bixby and Jhabvala 1990) but is inhibited by PTKs (Bixby and Jhabvala 1992). Since CAMs and ECM molecules utilize different kinases for the promotion of neurite outgrowth, they may also have very different effects on the cytoskeleton.

A variety of actin-binding proteins have been isolated and shown to have a seminal role in f-actin polymerization, cross-linkage of actin filaments and interactions between f-actin and the membrane (reviewed in Forscher 1989). The function of several brain-derived actin binding proteins such as gelsolin, fodrin, adducin, synapsin, and tropomyosin is markedly affected by alterations in intracellular calcium concentration \([Ca^{++}]_i\). Therefore, changes in \([Ca^{++}]_i\) could directly regulate the actin cytoskeleton. Another actin binding protein, profilin, acts as a buffer of actin monomers, and provides actin for preferential polymerization at the barbed ends of actin filaments. The site on profilin that binds actin also binds polyphosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). PIP\(_2\) has been shown to directly compete with actin for this binding site, and may play a role in cytoskeletal remodeling (Forscher 1989). It is interesting that the triggering of L1 or N-cadherin on the surface of cells has been shown to alter intracellular calcium, IP\(_2\), and IP\(_3\) levels (Schuch, Lohse et al. 1989; Halbach, Taylor
Therefore, cellular contact with L1 or N-cadherin could be transduced into an intracellular signal that directly affects cytoskeletal-associated proteins and results in cytoskeletal remodeling.

To explain the results observed here, the models shown in Figure 13 are proposed. In short, each of the substrate-receptor binding interactions elicits specific signals within the growth cone that result in distinctive morphology changes. The integrin receptor, upon binding laminin, has been shown to cluster in specialized junctions between the cell and substratum called focal adhesions in non-neuronal cells (Burridge, Fath et al. 1988). A number of proteins have been localized to focal adhesions by indirect immunofluorescence microscopy. These include vinculin, alpha actinin, and talin which, based on in vitro binding studies, are thought to link the integrin receptor to the actin cytoskeleton (Gumbiner 1993). The attachment of actin filaments to focal adhesions may be important for the force generation necessary for motility. In support of this idea, migrating fibroblasts have small focal adhesions at their leading edge which correlate with cell movement (Rinnerthaler, Geiger et al. 1988). Although focal contact-like structures have not been observed in growth cones (Gundersen 1988), focal contact associated proteins have been localized to growth cones (Letourneau and Shattuck 1989; Sobue and Kanda 1989), and in conjunction with other actin binding proteins, could potentially be involved in the formation of an integrin-associated actin network in growth cones.

L1/8D9 binding on the growth cone surface may result in an intracellular signal which originates from the cytoplasmic domain of the receptor, and is conveyed to the cytoskeleton by cytosolic proteins. Recently, an L1/8D9-like molecule (Davis, McLaughlin et al. 1993) has been shown to associate via its cytoplasmic domain with the cytosolic protein ankyrin that in turn interacts with the actin cytoskeleton. Under these conditions, the possibility exists that several collateral interactions with other
cytoskeletal proteins may also occur. For example, ankyrin interacts with spectrin for actin binding (Bennett 1990; Bennett 1992), but has also been shown to associate with tubulin directly (Davis and Bennett 1984; Davis, Otto et al. 1991) and via a spectrin-tau-tubulin interaction (Carlier, Simon et al. 1984). In addition, spectrin and intermediate filaments coprecipitate from cells injected with spectrin antibody (Mangeat and Burridge 1984). Therefore, growth cone interaction with substrate-bound L1/8D9 could potentially result in global cytoskeletal changes that are not limited to the actin cytoskeleton.

The localization of N-cadherin in cells by immunocytochemical methods corresponds exactly with cortical actin bundles (Hirano, Nose et al. 1987). It has been proposed that extracellular binding of N-cadherin results in a signal that is transmitted intracellularly, resulting in the activation of cytosolic components that could in turn bridge an interaction between N-cadherin and the actin cytoskeleton. These interactions are deemed necessary for a structural and functional network (Takeichi 1990). At the inner surface of cell membranes, the cytoplasmic tail of cadherins associates with the catenin proteins, alpha catenin, beta catenin and gamma catenin (Hirano, Kimoto et al. 1992; Gumbiner 1993; Kemler 1993) and is required for cadherin function (Ozawa, Ringwald et al. 1990). Alpha catenin is necessary for the adhesive function of cadherins and for organization of multicellular structures (Hirano, Kimoto et al. 1992). Alpha catenin shares similar structure and sequence homology with vinculin (Herrenknecht, Ozawa et al. 1991; Nagafuchi, Takeichi et al. 1991), a focal adhesion protein, and may therefore provide the interaction between N-cadherin and the actin cytoskeleton via other cytosolic proteins such as alpha actinin. If the cytoplasmic domain of N-cadherin does interact with the cytoskeleton, it is feasible that the binding of N-cadherin on the growth cone surface could result in substantial changes in actin distribution that are then converted into directional growth influences.
From this and other studies, it is evident that CAMs and ECM molecules can have very different influences on growth cone motility and neurite outgrowth. Also, the mechanisms by which growth cones interpret these influences are slowly becoming apparent. The original idea that CAMs and ECM molecules act only as growth-promoting substrates is clearly too simplistic. Growth cone pathfinding is a highly complex process that involves several extracellular cues and intracellular mechanisms in concert. Only after continued study on the direct influence of CAMs and ECM molecules on growth cone motility will the processes underlying appropriate pathfinding become clear.
Figure 1. Distribution of f-actin in growth cones on different substrates. Growth cones on L1/8D9 (a), laminin (b) or N-cadherin (c, d) were fixed and stained with rhodamine-phalloidin for visualization of f-actin. Note the f-actin meshwork isolated in the peripheral portion of the growth cone on L1/8D9 (a) in comparison with the thick f-actin bundles throughout the growth cone on laminin (b). F-actin in growth cones on N-cadherin was present as either a peripherally located meshwork (c) or as thick bundles associated with a meshwork throughout the growth cone. Calibration bar=10μm.
Figure 2. Quantification of the observed f-actin distribution. Growth cones growing on laminin, L1/8D9 or N-cadherin were stained with rhodamine-phalloidin for visualization of f-actin. The distribution of f-actin was visually tallied in fifty random growth cones per substrate in four separate experiments. The percent of the total growth cones with f-actin distributed at the peripheral edge (cross-hatched bars) versus those with f-actin throughout the growth cone (dotted bars) were plotted for each substrate. Comparison was made using the unpaired student’s t-test and the Fisher PLSD test; ** p<0.0001.
Figure 3. Distribution of f-actin in growth cones at a border between laminin and L1/8D9. Growth cones growing on dishes coated with alternating lanes of laminin and L1/8D9 were fixed and stained with rhodamine-phalloidin for visualization of f-actin. A small amount of fluorescein-labeled BSA was added to the L1/8D9 prior to coating the dish for localization of the border between substrates. Growth cones initiating on L1/8D9 displayed f-actin that was concentrated mainly in the peripheral portion of growth cones (a), but following laminin contact, the f-actin redistributed to fill the growth cone as thicker bundles (b). Growth cones initiating on laminin displayed f-actin in thick bundles throughout the growth cone (c) that redistributed to the periphery following extensive contact with L1/8D9. Calibration bar=10µm.
Figure 3. Distribution of f-actin in growth cones at a border between laminin and L1/8D9.
Figure 4. Distribution of f-actin in growth cones at a border between laminin and N-cadherin. Growth cones growing on dishes coated with alternating lanes of laminin and N-cadherin were fixed and stained with rhodamine-phalloidin for visualization of f-actin. A small amount of fluorescein-labeled BSA was added to the N-cadherin prior to coating the dish for localization of the border between substrates. Growth cones initiating on N-cadherin displayed f-actin in a meshwork that redistributed to fill the entire growth cone after extensive leading edge contact with laminin (a, c). Once the central portion of the growth cone crossed onto laminin, the f-actin redistributed to thick bundles that filled the growth cone (b). Growth cones that crossed onto N-cadherin from laminin displayed f-actin concentrated as a meshwork in the peripheral region of the growth cone (d). Calibration bar=10μm.
Figure 4. Distribution of f-actin in growth cones at a border between laminin and N-cadherin.
Figure 5. Distribution of microtubules in growth cones growing on different substrates. Growth cones growing on L1/8D9 (a), N-cadherin (b) or laminin (c) were fixed and stained with the YL-1/2 antibody against tubulin. Note the loose pattern of gently curving microtubules that penetrate the periphery of growth cones on L1/8D9 (a) and N-cadherin (b). This pattern contrasts with the more tightly organized bundles of microtubules that remain confined to the central portion of growth cones on laminin (c). Calibration bar=10 μm.
Figure 5. Distribution of microtubules in growth cones growing on different substrates.
Figure 6. Distribution of microtubules in growth cones at a border between laminin and L1/8D9. Growth cones growing on dishes coated with alternating lanes of laminin and L1/8D9 were fixed and stained with the YL-1/2 antibody against tubulin. A small amount of rhodamine-labeled BSA was added to the L1/8D9 prior to coating the dish for localization of the border between substrates. The microtubules in growth cones initiating on laminin were present as relatively tight bundles that were often observed to reorient toward the site of growth cone contact with the L1/8D9 (a). As a larger region of the leading edge contacted L1/8D9, the microtubules became more loosely associated (b). Once the central portion of the growth cone crossed onto the L1/8D9, the microtubules splayed out into more individual filaments (c). Calibration bar=10μm.
Figure 6. Distribution of microtubules in growth cones at a border between laminin and L1/8D9.
Figure 7. Distribution of microtubules in growth cones at a border between laminin and N-cadherin. Growth cones growing on dishes coated with alternating lanes of laminin and N-cadherin were fixed and stained with the YL-1/2 antibody against tubulin. A small amount of rhodamine-labeled BSA was added to the N-cadherin prior to coating the dish for localization of the border between substrates. The microtubules in growth cones initiating on N-cadherin were loosely associated and often splayed apart. Upon initial contact with laminin, growth cone microtubules reoriented toward the site of contact (a). Upon more extensive contact with laminin, microtubules became more tightly associated and confined to the central portion of the growth cone (b). Growth cones that crossed onto N-cadherin from laminin displayed microtubules throughout the growth cone that were loosely associated and splayed out into the periphery (c). Calibration bar=10μm.
Figure 7. Distribution of microtubules in growth cones at a border between laminin and N-cadherin.
Figure 8. Distribution of neurofilaments in growth cones growing on different substrates. Growth cones growing on L1/8D9 (a, d), N-cadherin (b) or laminin (c) were fixed and stained with the 5E10 antibody against neurofilament. In growth cones on L1/8D9, the neurofilaments occurred as loosely associated, straight bundles that filled the central region of the growth cone (a). Neurofilaments in growth cones on N-cadherin also filled the central portion of the growth cone, but occurred as loosely associated, curved filaments (b). In growth cones on laminin, the neurofilaments ended abruptly at the base of the growth cone in a tight bundle (c), and were rarely observed to enter the central portion of the growth cone. When two growth cones growing on either L1/8D9 or N-cadherin were in contact with one another, neurofilaments often reoriented toward the adjacent growth cone (d). Calibration bar=10μm.
Figure 8. Distribution of neurofilaments in growth cones growing on different substrates.
Figure 9. Distribution of neurofilaments in growth cones at a border between laminin and L1/8D9. Growth cones growing on dishes coated with alternating lanes of laminin and L1/8D9 were fixed and stained with 5E10 antibody against neurofilament. The dotted line indicates the border between substrates, and the arrowheads indicate the leading edge of each growth cone. Neurofilaments in growth cones initiating on L1/8D9 were loosely associated and penetrated into the central portion of the growth cone (d). Upon extensive growth cone contact with laminin, neurofilaments became more tightly associated and did not penetrate as far into the central region of the growth cone (a, b). Growth cones initiating on laminin maintained tightly associated neurofilaments after leading edge contact with L1/8D9 (c), that became more loosely associated only after the central portion of the growth cone crossed onto L1/8D9 (not shown). Calibration bar=10μm.
Figure 9. Distribution of neurofilaments in growth cones at a border between laminin and L1/8D9.
Figure 10. Distribution of neurofilaments in growth cones at a border between laminin and N-cadherin. Growth cones growing on dishes coated with alternating lanes of laminin and N-cadherin were fixed and stained with 5E10 antibody against neurofilament. The dotted line indicates the border between substrates, and the arrowheads indicate the leading edge of each growth cone. Neurofilaments in growth cones initiating on N-cadherin were loosely associated in the central portion of the growth cone. As the growth cone began crossing onto laminin, the neurofilaments spread along the border between substrates, remaining in the portion of the growth cone on N-cadherin (a, b). Once the growth cone completed crossing onto laminin, the neurofilaments became confined in a tighter bundle to the central portion of the growth cone more proximal to the neurite (c). Growth cones initiating on laminin that contacted N-cadherin with their leading edge maintained neurofilaments in a tight group of bundles localized to the base of the growth cone (d). Calibration bar=10μm.
Figure 10. Distribution of neurofilaments in growth cones at a border between laminin and N-cadherin.
Figure 11. Distribution of phosphotyrosine, p59<sup>fyn</sup> or pp60<sup>src</sup> in growth cones growing on different substrates. Growth cones growing on L1/8D9 (a, b, c), N-cadherin (d, e, f) or laminin (g, h, i) were fixed and stained with antibodies against phosphotyrosine (a, d, g), p59<sup>fyn</sup> (b, e, h) or pp60<sup>src</sup> (c, f, i). Note the similar punctate staining pattern for each antigen throughout the entire growth cone and into the filopodia on each substrate. Calibration bar=10μm.
Figure 12. Distribution of phosphotyrosine in growth cones at a border between laminin and L1/8D9 or N-cadherin. Growth cones growing on dishes coated with alternating lanes of laminin and L1/8D9 (a, b, d) or laminin and N-cadherin (c) were fixed and stained with the 4G10 antibody against phosphotyrosine. A small amount of rhodamine-labeled BSA was added to the L1/8D9 or N-cadherin prior to coating the dish for localization of the border between substrates. In all substrate combinations examined, the punctate staining for phosphotyrosine remained unchanged in intensity or pattern. Calibration bar=10µm.
Figure 12. Distribution of phosphotyrosine in growth cones at a border between laminin and L1/8D9 or N-cadherin.
Figure 13. Model of the possible L1, N-cadherin or Laminin mediated events in the growth cone. L1 mediated growth (a) could result in direct influences on the actin cytoskeleton via an L1-ankyrin-spectrin-actin interaction or an L1-ankyrin-microtubule interaction (Davis and Bennett 1984; Davis, McLaughlin et al. 1993). L1 binding has also been shown to activate multiple signaling pathways for neurite outgrowth that include phospholipase C (PLC), diacylglycerol (DAG), arachidonic acid (AA), G proteins, and an involvement of N- and L-type calcium channels. These signals may be generated due to a cis interaction with the FGF receptor (Doherty and Walsh 1994; Williams, Furness et al. 1994). A tyrosine kinase such as pp60^src appears to have a partial role in neurite outgrowth (Igelzi, Miller et al. 1994). In addition, L1 has been shown to associate with casein kinase II (CKII) and another yet undefined L1 kinase (L1k), but the function of these molecules is unclear (Sadoul, Kirchhoff et al. 1989; Schaefer, Wong et al. 1993). N-cadherin mediated growth (b) could result in alteration of the actin cytoskeleton via an α-catenin and α-actinin interaction (Hirano, Nose et al. 1987; Takeichi 1990). N-cadherin binding activates multiple intracellular signaling pathways, similar to those activated by L1, that are required for neurite outgrowth (Doherty and Walsh 1994). The generation of these signals may also require a cis interaction with the FGF receptor (Williams, Furness et al. 1994). Laminin mediated growth (c) may be due to an interaction of the integrin receptor with the actin cytoskeleton via proteins that have been localized to focal adhesions, such as vinculin, talin, and α-actinin (Sobue and Kanda 1989). Neurite outgrowth on laminin appears to require protein kinase C (PKC), but the signaling pathway activated by laminin is distinct from those activated by CAMs (Bixby 1989; Doherty and Walsh 1994). In non-neuronal cells, phosphorylation of the integrin receptor or focal adhesion proteins regulates their interaction and binding specificities (Romer, Burridge et al. 1992), but this has not been demonstrated directly for neurons.
Figure 13 a. L1 mediated events
Figure 13 b. N-cadherin mediated events

Legend:
- Actin Filament
- Microtubule
- Alpha Catenin
- Beta Catenin
- Alpha Actinin
- N-cadherin

Diagram showing the interaction of various cellular components, including G proteins, PLA₂, PLC, DAG, AA, Ca²⁺, and neurite outgrowth.
Figure 13 c. Laminin mediated events
LITERATURE CITED


CHAPTER 5

Concluding Statements
The results described in this thesis have provided evidence that substrate-bound CAMs and ECM molecules are capable of inducing very different responses from growth cones, exhibited in the form of behavioral changes as well as alterations in the intracellular cytoskeletal components. The results are intriguing because they suggest that these molecules are capable of actively altering growth cone motility in ways that could be important during pathfinding. Although the molecules tested here all promote cell adhesion and neurite outgrowth, they most likely have additional distinct functions that have yet to be described. The recent demonstration of signal transduction pathways associated with CAM or ECM receptor binding supports this notion, and adds to the complexity of events mediated by these molecules.

An interaction between the CAM or ECM receptor and the growth cone cytoskeleton, either directly through protein-protein interactions or indirectly via intracellular signaling pathways, has enormous implications. For example, growth cone contact with these molecules could promote stabilization of the cytoskeleton, resulting in a directional change toward the site of stabilization. This type of scenario could be important at choice points in the embryo. In contrast, a complete rearrangement of the cytoskeleton, such as that which occurs during a growth cone collapse, could be induced to allow the growth cone to change its cytoskeletal structure to one appropriate for the new CAM or ECM molecule. This sort of mechanism would allow a growth cone to quickly adapt to a changing environment in the embryo. Future efforts aimed at understanding how CAM or ECM receptors interact with the cytoskeleton will resolve these issues.

In the developing embryo, each growth cone encounters a multitude of different CAMs and ECM molecules as it progresses toward its target tissue. At present, it is unclear how growth cones discriminate between molecules that are encountered simultaneously. Is it possible that specific intracellular signals are somewhat dominant
over others? Under these conditions, a signal generated by the binding of one type of CAM could override or quench signals derived from other CAM-mediated events. An example of this could be a signaling pathway that culminates in a global increase in intracellular calcium concentration. This scenario would result in a behavioral response to the dominant CAM signal, while the other CAM-mediated signal(s) would be without effect. While this is an intriguing idea, it also demonstrates the complexity of potential interactions that occur within the growth cone.

CAM-generated signals are clearly necessary for the general promotion of neurite outgrowth, but it is unclear whether separate signals also underlie the distinct growth cone morphology and behavioral changes that are induced by different CAMs and ECM molecules. Many of the elements in the growth cone locomotory machinery are regulated by post-translational modifications such as phosphorylation. A CAM-derived signal that generates kinase or phosphatase activity could potentially influence the cytoskeleton and ultimately motility. Therefore, it is important to identify these separate signals in order to fully comprehend the scope of effects that CAMs and ECM molecules produce in growth cones. Only once this is accomplished will it be possible to gain a full understanding of how growth cone motility is influenced by specific CAMs and ECM molecules in the developing embryo.


