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The regulated function of integrins and cell adhesion molecules in retinal neuron development

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THE REGULATED FUNCTION OF INTEGRINS AND CELL ADHESION MOLECULES IN RETINAL NEURON DEVELOPMENT

by

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DISSERTATION

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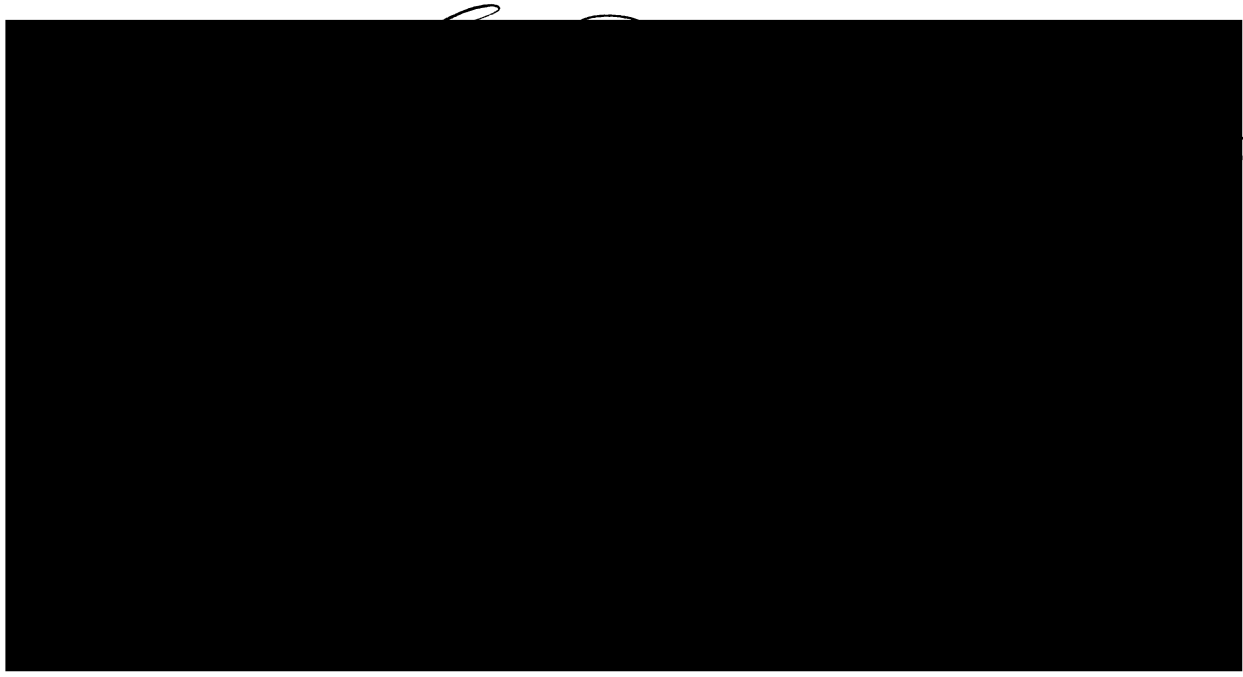

For Joe Howard

ACKNOWLEDGEMENTS

The process of seeing this thesis to its end has brought me face to face with a number of paradoxes, some of which concern science. One of the most profound, however, is that at the climax of one's appreciation for one's friends, one has to leave them -- at least geographically. I was recently searching for a crossword puzzle clue in Thoreau's Walden, and I came upon a paragraph that I had marked with stars and lines ten years ago as an undergraduate. He was writing about building a house in New England, but I thought he might as well have been writing about twentieth century Science:

> Most men, even in this comparatively free country, through mere ignorance and mistake, are so occupied with the factitious cares and superfluously coarse labors of life that its finer fruits cannot be plucked by them. Their fingers, from excessive toil, are too clumsy and tremble too much for that. . . . He has no time to be anything but a machine. How can he remember well his ignorance -- which his growth requires -- who has so often to use his knowledge? We should feed and clothe him gratuitously sometimes, and recruit him with our cordials, before we judge of him. The finest qualities of our nature, like the bloom on fruits, can be preserved only by the most delicate handling. Yet we do not treat ourselves nor one another thus tenderly.

Another paradox then became clear. While the scientific process/community bespeaks a dearth of humility and tenderness that would, in fact, foster itself, individuals within the system break the rules with their openness of mind and heart. These are the qualities that I acknowledge in the people who have shaped my experience:

Louis Reichardt, my advisor, whose intellect has been an inspiration and a guide.

During my first months in the lab, Louis taught me how to run a gel and to stain cells. Working closely with him at the bench, I first became infected with the desire to understand how axons grow. In my naivete, I did not realize that at the end of six years I would still be ignorant on this question. But it doesn't bother me too much, because Louis has shown me the simple rewards in the asking of the question and in the design of experiments that go to the heart of each issue. Throughout, Lou's vote of confidence helped to smooth an otherwise rocky road.

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Kevin Jones, who taught me to throw a frisbee and to catch it (sometimes in the teeth), running at full speed in the dark. He invented the PhD machine and coined my many nicknames, some of them derogatory. Our periods of frantic bench-work fortunately coincided and led to many intense discussions of our work. His contributions, including his part in the flickering-tongue model of integrin function, were indispensible.

Outside of the lab, Bruce Hay has been a long-time friend and companion. We went through orals and thesis-, paper-, and fellowship-writing together, and I consider myself the beneficiary. Bruce gives all of his attention to the science at hand and never asks a stupid question.

The members of my thesis committee, Caroline Damsky, Zach Hall, Scott Fraser, and Lily Jan, took turns at being supportive and enthusiastic. Each made valuable contributions to the writing of various chapters and kept her/his sense of humor even when things weren't very funny.

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I am also endebted to my family and friends whose history predates my graduate career. My parents, each in their own way, believed I could do IT. In particular, my mother Nan put all of her eggs in one basket when she had me, and ever since has invested in my potential in whatever ways she could. My undergraduate advisor, Miriam M. Salpeter, deserves the incommunicable thanks that result from her having first interested me in the doing of science and from her having fostered my economic survival at Cornell.

This thesis is dedicated to Jonathon "Joe" Howard who was the chocolate at the end of my PhD machine. His approach to science has greatly influenced mine, although I have much still to learn from him. So far, he has taught me the importance of the testable

hypothesis and to be as intellectually stringent as existing knowledge permits. Knowing Joe as a scientist has been an opportunity to observe clear thinking in action, and I hold that clarity as a standard for myself. In addition, Joe's love and companionship has been my buoy for five years. My life is full of the richness of our past, the joy of our present, and the anticipation our future together.

Finally, I want to acknowledge our species for having created music and our nervous system for having evolved to respond to it. Music has helped to sustain me, especially in the last year. My friends will perhaps be surprised that (rather than the Talking Heads' "Burning Down the House") the most important piece of music to me is Franz Schubert's "Great" Symphony #9 in C Major, the rhythms of which secretly punctuate the verbiage that follows.

THE REGULATED FUNCTION OF INTEGRINS AND CELL ADHESION MOLECULES IN RETINAL NEURON DEVELOPMENT

Karla M. Neugebauer

ABSTRACT

During development, neurons extend axons and dendrites through extracellular space to contact synaptic partners some distance away from the cell body. The appreciation that pathfinding is highly specific and that growth cones must adhere to move has led to the prediction that process growth depends on the activity of specific cell surface receptors which bind molecular cues in the environment. What are the molecules that regulate neuronal adhesion and process extension, and how is receptor activity regulated?

Embryonic day 7 (E7) chick retinal neurons attached and extended neurites on astrocyte monolayers and substrates coated with the following purified extracellular matrix (ECM) proteins: laminin, fibronectin, collagens, thrombospondin, and vitronectin. Antibodies to members of the integrin family of ECM receptors inhibited responses to each of these substrates. In addition, a heparan sulfate proteoglycan(s) was implicated in retinal neuron adhesion to thrombospondin, and responses to astrocytes were mediated, in large part, by two cell adhesion molecules, N-cadherin and NCAM. Interestingly, antibodies that had profound effects on neurite elongation on astrocytes (eg. anti-N-cadherin) did not reduce cell adhesion to the monolayer, suggesting that "adhesive" receptors must transduce a distinct signal for neurite outgrowth.

The activities of almost all of these receptors were developmentally regulated in two ways: (i) Retinal neurons from older embryos (E11) exhibited a complete loss of integrin function, since they no longer attached or grew neurites on laminin or fibronectin. However, adhesion to laminin was induced by the monoclonal antibody

TASC which was shown to bind and activate the function of the integrin β_1 subunit. Thus, it appears that functional regulation of integrins includes a reversible inactivation event at the cell surface. (ii) Adhesion was separable from neurite outgrowth for two classes of receptors. NCAM promoted adhesion but not neurite outgrowth at E7, while NCAM played a major role in E11 neurite outgrowth on astrocytes. Conversely, E11 retinal neurons continued to adhere to collagens, vitronectin, and thrombospondin but did not extend neurites. Three functional states of receptors are suggested: (1) expressed but inactive, (2) expressed and able to mediate cell adhesion, and (3) expressed, adhesion-competent and able to promote neurite outgrowth. Receptors apparently interconvert between receptor states, providing a dynamic mechanism for regulating cell interactions.

Thus, multiple receptors promote cell adhesion and neurite outgrowth, and many are subject to functional modulation. The particular receptors expressed, their activation state, and the distribution of their ligands in the environment are likely to determine the pathways that axons and dendrites choose in vivo.

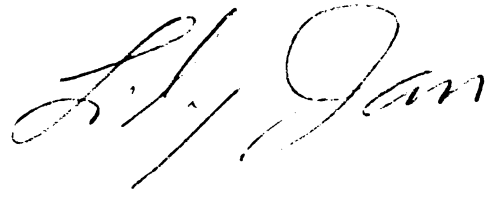
Chairman: 

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES Page

CHAPTER ONE

INTRODUCTION

One of the earliest questions to be asked about the nervous system was whether this anatomically diffuse organ is composed of discrete cells that connect in ways allowing for the transmission of electrical impulses or rather a syncitium of protoplasm with similar capabilities. The answer to this question would have dramatically different ramifications for understanding how the nervous system develops. Had the latter "reticular" theory proven true, the job of developmental neurobiologists would have been perhaps to explain how strings of cells or even non-cellular protoplasm fuse to produce axons, much as developmental biologists are still trying to explain the genesis of multinucleate muscle fibers. Instead, the work of Wilhelm His, Ramon y Cajal and contemporaries favored the former "neuron" theory (Cajal, 1954), leading to a series of questions surrounding the requirement that, once born, neurons must elaborate axons and dendrites through a virtual maze of neural and non-neural tissue to make meaningful connections with each other and with effector cells. The neuron versus reticular theory controversy raged for some time, and it is interesting to note that Cajal's appreciation that growth cones represent the motile tips of developing axons helped him to form his argument while providing a first clue to the mechanisms that lead to axon growth and guidance (Cajal, 1929; 1954).

Early observers of growth cone behavior in vivo and in vitro were quick to consider the physical factors that might underly its "ameboid" movement. Mechanical or structural influences (thigmo- or stereotropism), chemical stimuli (chemotropism), and electrical activity (galvanotropism) were all invoked and persist in current concepts of axonal growth and guidance. The notion of stereotropism stems from the observation that growth cones and the axons "spun" behind them follow objects in their environment. In tissue sections, neurites were founding "resting" on fibroblasts, other cells, or anatomical structures (Cajal, 1929). In vitro, growth cones followed the filaments of a spider's web (Harrison, 1935).

Speidel (1933) made the first in vivo time lapse study of growth cone behavior, using the translucent tadpole tailfin, and described the formation of axon fascicles whereby growth cones "glide along the pioneer fiber" that had gone before it. Cajal (1929) had the most to say about chemical cues, calling on them to explain, for example, the directed growth of optic axons towards the optic nerve. Seemingly pure intuition led him to write:

> From the functional point of view the growth cone may be regarded as a sort of club or battering ram, endowed with exquisite chemical sensitivity, with rapid amoeboid movements, and with a certain impulsive force, thanks to which it is able to press forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination (Cajal, 1899, quoted in Harrison, 1935).

In apparent deference to their colleagues, Harrison and Speidel considered the potential for electrical activity to influence growth but were at a loss to provide that evidence themselves. Largely due to the experiments of these two, therefore, attention was focused in the years to come on mechanical and chemical means of achieving growth cone motility.

Over the years, mechanical and chemical themes have become fused in our thinking about growth cone motility. This is due to the appreciation of several facts. First, growth cone motility reflects, as does muscle contraction, the conversion of chemical to mechanical energy within the growth cone. Second, growth cones exert a tensive force on the neurite behind it, and necessarily must be attached to a relatively stationary object to do so. Third, this attachment of the growth cone must be mediated by molecules in the plasma membrane which interact with molecules present on the substrate be it biological or artificial.

Molecules and the Specification of Axonal Growth

With the premise that growth cone motility and subsequent axon elongation are the products of mechano-chemical events performed by molecules, another

philosophical step must be taken to infer the action of specific receptors on the surface of the growth cone which bind specific ligands in the environment. This idea derives from early attempts to explain, in chemical terms, the specificity with which neurons innervate their targets. Cajal (1929) invokes a gradient of "chemotactic material" in the optic pathway to explain the directed growth of retinal ganglion cell axons. Paul Weiss (1936) proposed a chemical contribution to the specificity of muscle innervation in his classic studies of limb transplantation in the salamander. The first soluble chemotactic molecule, nerve growth factor (NGF), was identified in the 1950's and shown to orient axon growth when exogenously added to developing embryos (cf. Levi-Montalcini, 1976), providing an important precedent for the existence of specific guidance molecules. These ideas developed in parallel with nascent molecular theories regarding the specificity of cell-cell adhesion during morphogenesis, in particular the differential cell adhesion hypothesis (cf. Weiss, 1961; Steinberg, 1970). In 1963, Sperry contributed his chemoaffinity hypothesis which explicitly called for molecular labels to match each axon with its central target in the development of the primary optic pathway. It is clear that Sperry overstated his case, since the pairing of each afferent neuron with its target neuron has been shown to be plastic. Nevertheless, Sperry's appreciation of the usefulness of molecules in specifying neural connections was critical to the intellectual development of the field.

The past two decades have seen an explosion of biochemical support for the proposal that axon growth and guidance are receptor mediated. Indeed, it has virtually been made dogma by the identification of molecules in the environment and their neuronal receptors which, acting together, elicit neurite outgrowth in vitro. However, we do not yet fully understand the roles of receptors and their ligands in process initiation, growth cone motility, pathfinding, or synaptogenesis, especially in vivo. Instead, we must confess that, in most cases, we infer the activity of

receptors and ligands, using a circular argument that goes like this: a) The activities of molecule X and its receptor Y promote neurite outgrowth in vitro, and b) Since molecules X and Y are present in pathway P, they must function in pathfinding in vivo. The order of a and b can be reversed, and permutations of this logic have been used in theorizing about the generation of neuronal polarity and the behavior of growth cones in vivo and in vitro. Obviously, the use of in vitro model systems has been indispensible to the identification of molecules that *can* be operative in the development of the nervous system. But, armed with the ensuing bundles of data, we cannot promulgate our now favorite theory of receptor-mediated growth cone guidance without the caution that a number of requirements must be filled before we believe that molecules X and Y function in the development of pathway P.

What must a molecule do to be accepted as an element in the process of axonal growth and guidance? Some molecules, like the extracellular matirx (ECM) glycoprotein laminin, can be purified and shown to promote neurite outgrowth by isolated neurons in vitro (Manthorpe et al., 1983; Lander et al., 1985). If laminin were not active in such cell culture experiments, one could not conclude that laminin does not promote growth in vivo, since laminin may have different activities when assembled into basement membranes. Indeed, many such studies are carried out with laminin purified from heterologous tissue sources, such as the murine EHS sarcoma, and it is now known that molecular variants of laminin are expressed in different tissues (Hunter et al., 1989; Ehrig et al., 1990). Likewise, the fact that laminin is active in vitro does not immediately mean that it functions in vivo. Indeed, other components of basement membranes and even some forms of laminin itself (eg. S-laminin) may modify or even inhibit the neuronal response to laminin (Sandrock and Matthew, 1987b; Hunter et al., 1990). Fortunately, the observation that a monoclonal antibody (INO) against a laminin-heparin sulfate proteoglycan complex inhibits the regeneration of sympathetic nerve fibers into rat

irides in situ provides more direct evidence that laminin actually acts to promote axonal growth in vivo (Sandrock and Matthew, 1987a).

The above consideration of laminin illustrates the possibility that molecule X may or may not speak the same language to a given neuron in vivo as it does in vitro. Inasmuch as the regulation of axonal growth and guidance by receptors and ligands is a question of molecular semantics, clear terminology is essential to this discussion. *Growth cone motility* denotes the movement of the tip of a neuronal process (axon or dendrite), including the extension or retraction of filopodia and lamellopodia coupled to a change in the position of the growth cone with respect to the cell body. Growth cone motility is considered to be a prerequisite for *pathfinding*, a more holistic term which describes the directed growth of a neuronal process, including its behavior at "choice points" along the pathway and the decision to stop at the target. Pathfinding per se does not include subsequent events of synaptogenesis or competitive interactions between axon terminals and their targets.

In considering receptor-mediated growth cone motility and guidance, the primary molecules of interest are naturally the receptors expressed on the surface of the growth cone and their molecular ligands in the environment. Secondary to these, but no less important, are the molecules inside the growth cone that regulate its motility in response to ligand binding. These include a diverse set of molecules from free ions (eg. calcium), to second messenger compounds (eg. cAMP, IP3), to regulatory enzymes (eg. kinases, proteases), to cytoskeletal proteins that link receptors to the actin-rich cortex of the growth cone. It is useful to establish a few criteria for the molecules that mediate growth cone motility and guidance. For the receptors and their ligands, these include:

1) The receptor must be expressed on the surface of the growth cone, and the ligand must be expressed in its observed path or at the target.

2) Loss or gain of function of either molecule must result in reduced, enhanced, or differently oriented growth. While the use of cell culture systems can help to identify ligands and receptors with molecular precision, only studies in vivo or at least approximating in vivo situations (eg. tissue sections, organ culture systems) can directly implicate a pair of molecules in pathfinding.

The Case for Receptor-Mediated Growth Cone Motility

The growth cone, most distal to the neuronal cell body, is the first part of the axon or dendrite to encounter new environments and therefore new molecular signals. The insertion of new plasma membrane actually occurs at the growth cone, allowing for the material increase in neuronal process length (Bray, 1970; Cheng and Reese, 1987). At the same time, the growth cone exerts tension on the shaft of the growing process, as has been demonstrated by direct physical measurements (Bray, 1979; Dennerll et al., 1988; Lamoureux et al., 1989). Remarkably, processes can be induced to grow by pulling on the plasma membrane of neuronal cell bodies, consistent with the view that tension exerted by the growth cone plays a direct role in the elaboration of the process behind it (Bray, 1984). In vitro, the growth cone autonomously determines neuronal morphology, including neurite branching (Bray, 1973). Thus, growth cone activity is implicated in every aspect of process extension. What molecular mechanisms underly growth cone function and do predictions arise for a regulatory role of receptors?

The current consensus on the molecular basis of growth cone motility is founded in growth cone anatomy, the observed distribution of cytoskeletal elements, and growth cone behavior observed in vitro (reviewed in Mitchison and Kirschner, 1988; Smith, 1988). The growth cone consists of three distinct domains that be compared to the form of a human hand. The extremity of the growth cone is populated by filopodia, protrusive "fingers" of filamentous actin that are rapidly

extended and retracted (cf. Bray and Chapman, 1985). An obvious function of the filopodium is to efficiently expand the range of environmental exploration possible to the growth cone. The structure of the core or "palm" of the growth cone is dominated by a meshwork of filamentous actin less organized than the actin bundles found in filopodia (Yamada et al., 1971). The leading edge of the palm region is called the lamellopodium, and the directed flow of this structure followed by the influx of cytoplasm and organelles is likely to determine the net movement of the growth cone as a whole (Goldberg and Burmeister, 1986; Aletta and Greene, 1988). At the transition zone between the palm and the neurite shaft (the "wrist") assembled microtubules of the neurite interface with the actin-rich cortex of the growth cone. Substantial assembly of microtubules occurs at this site and is required for the elongation of the neurite (Shaw and Bray, 1977; Bamburg et al., 1986). Nevertheless, the integrity of actin-based motility is required for the generation of tension in the neurite and the elongation of the neuronal process (Yamada et al., 1970; Shaw and Bray, 1977; Dennerll et al., 1988). Thus, the interplay between the molecules that comprise the three regions of the growth cone must determine its behavior.

The current view of growth cone motility holds that forward motion of the growth cone is produced by a "cortical flow" mechanism, whereby filamentous actin is driven in the retrograde direction by molecular motors, most likely myosin and its isoforms (Bray and White, 1988; Mitchison and Kirschner, 1988). Whereas such cortical flow alone would result in a futile cycle of cytoskeletal dynamics that would not produce net movement, forward displacement of the growth cone is generated when growing actin filaments resist the forces exerted by myosin. This resistance is achieved by the attachment of the growth cone to the substratum and the physical linkage of actin filaments to the now immobilized plasma membrane (Mitchison and Kirschner, 1988). Thus, growth cone adhesion is a prerequisite for

net forward movement as is the existance of molecular links between actin filaments and the plasma membrane. Consistent with this model is the observation that myosin is present in filopodia and lamellopodia as are the actin-associated proteins, fodrin, talin, vinculin, filamin and α-actinin (Pollerberg et al., 1987; Letourneau and Shattuck, 1989; Bridgman and Dailey, 1989). The latter set of proteins are likely regulators of the actin linkage to the adhesive plasma membrane by virtue of their interactions with the cytoplasmic domains of specific transmembrane proteins.

Implicit in this treatment of growth cone motility is the specificity of the interaction between actin filaments and the plasma membrane. Clearly, there must be strict regulation of the actin-membrane relationship or a single lamellopodium would emerge, wave-like, from the entire perimeter of the cultured neuron. The transmembrane proteins likely to bear binding sites for actin-associated proteins are also likely to be the molecular elements that mediate the attachment of the growth cone to specific ligands, since attachment is required for movement. This prediction has been borne out in a number of cases, providing strong evidence for receptor-mediated growth cone motility at least in vitro. A list of the transmembrane proteins that promote neurite extension in vitro and their intracellular and extracellular ligands is presented in Table 1.1 and briefly summarized below. These include two classes of receptors, (1) members of the integrin family of ECM receptors that mediate neuronal interactions with secreted glycoproteins found in basal laminae and in extracellular spaces and (2) cell-cell adhesion molecules (CAMs) that mediate neuronal interactions with cell surfaces.

Historically, the ability of integrins and their ligands to promote neurite outgrowth was appreciated first, beginning with the purification of molecules that stimulated neurite outgrowth when adsorbed to the substratum. The identification of laminin, a trimolecular glycoprotein complex, was arguably the most exciting, since it could be purified from culture medium conditioned by relevant cell types such as

glial and muscle cells (cf. Davis et al., 1985; Lander et al., 1985; Calof and Reichardt, 1985) and since it promoted extensive process growth by both peripheral and central neurons (cf. Baron von Evercooren, 1982; Manthorpe et al., 1983; Rogers et al., 1983; Lander et al., 1983). Laminin has subsequently been localized to basement membranes and extracellular spaces in the developing peripheral and central nervous systems an in peripheral target tissues, consistent with its potential to regulate axonal growth and guidance in vivo (Rogers et al., 1986; Sanes et al., 1986; Cohen et al., 1987; Letourneau et al., 1988). Similar results have been obtained for additional ECM constituents, most notably collagens and fibronectin, although none of these generally elicits responses as exuberant as does laminin (Vlodavsky et al., 1982; Carbonetto et al., 1983; Rogers et al., 1983; Adler and Hewitt, 1985; Hall et al., 1987; Bixby, 1989). Fibronectin, in particular, has more potent neurite outgrowth-promoting effects on peripheral neurons than it does on central neurons, consistent with its more prominent and widespread distribution in peripheral tissues (Rogers et al., 1986; Sanes et al., 1986; Halfter and Fua, 1987).

The integrins are a family of cell surface receptors, initially identified on fibroblasts and cancer cell lines, that mediate cell interactions with a variety of ECM constituents and cell surface ligands (cf. Akiyama et al., 1990). Each integrin receptor is a heterodimer composed of one α and one β subunit, the particular combination of which determines the ligand-binding specificity of the receptor. To date, 12 α and 6 β subunits have been identified and shown to regulate cell adhesion, shape, motility and migration, differentiation, and gene expression (cf. Akiyama et al., 1990; Hemler, 1990; Reichardt and Tomaselli, 1991). The mechanisms underlying the pleiotropic cellular consequences of integrin binding are likely to include both cytoskeletal rearrangement and the regulation of second messenger systems. The actin-associated proteins, talin and α-actinin, have been shown to bind the cytoplasmic domain of the integrin β_1 subunit biochemically, and

α-actinin similarly interacts with the cytoplasmic domain of the integrin β_3 subunit (Horwitz et al., 1986; Otey et al., 1990). These observations, along with the colocalization of actin filaments, ECM molecules, and integrins at sites of cell-substratum contact (cf. Damsky et al., 1985), argue that integrins serve as a molecular link between the cellular environment and the cytoskeleton, modifying cell behavior in the process. In addition, integrin binding has been associated with the activation of gene expression and with signal transduction by second messengers and protein kinases in immune cells and neurons (cf. Werb et al., 1989; Bixby, 1989; Hemler, 1990; Springer, 1990).

Direct evidence that members of the integrin family correspond to the neuronal receptors that mediate neurite outgrowth on ECM constituents was obtained using function-blocking integrin antibodies. Initial experiments showed that integrin function was required for peripheral neurite outgrowth on laminin, fibronectin, and collagen (Bozyczko and Horwitz, 1985; Tomaselli et al., 1986). Subsequently, integrins that bind laminin and collagen were purified from neuronal cell lines (Tomaselli et al., 1988a; Ignatius and Reichardt, 1988). Each of these heterodimers was composed of the integrin β_1 subunit in non-covalent association with one of several α subunits, implicating members of the β_1 class of integrins in neuronal responses to laminin and collagen where encountered by neurons in vivo. Table 1.1 lists all of the known β_1-class integrin heterodimers, since each has relevant extracellular ligands. Although it is not yet clear that each $\alpha\beta_1$ dimer promotes neurite outgrowth, the activities of $\alpha_1\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ have been shown to be important in neurite outgrowth on laminin (Turner et al., 1989; K.J. Tomaselli, C.J. Emmett, P. Doherty, and L.F. Reichardt, unpublished observations; J. Cohen, unpublished observations). The likelihood that these integrins regulate growth cone motility, at least in part, by interacting with the actin microfilaments is supported by the observation that β_1-class integrins, talin, α-actinin and several

other actin-associated proteins are localized to the growth cone in vitro and to growing axons in vivo (Bozyczko and Horwitz, 1985; Cohen et al., 1987; Letourneau and Shattuck, 1989).

Purified ECM molecules are not alone in their ability to promote neurite outgrowth in vitro. Both peripheral and central neurons rapidly elaborate processes on muscle, astrocyte, and Schwann cell monolayers as well as on previously extended axons (Rutishauser et al., 1983; McCaffery et al., 1984; Noble et al., 1984; Fallon et al., 1985a and b; Chang et al., 1987). Importantly, neurite outgrowth on non-neuronal cell substrates is dependent on contact with the substrate cell and is not eliminated by anti-integrin β_1 antibodies (Tomaselli et al., 1986). This observation argues strongly that ligands expressed on non-neuronal cell surfaces function as neurite-promoting molecules.

As for integrins, the use of function-blocking antibodies against a variety of neuronal cell surface glycoproteins has identified a number of CAMs that account for the neurite outgrowth-promoting activity of cell surfaces (see Table 1.1). These include one member, N-cadherin, of the cadherin family of the Ca^{++}-dependent CAMs, and five members of the Ca^{++}-independent immunoglobulin superfamily: NCAM, L1 (aka NgCAM, NILE, G4), myelin-associated glycoprotein, F11/contactin, and TAG-1 (Small et al., 1987; Bixby et al., 1988; Hatta et al., 1988; Moos et al., 1988; Ranscht, 1988; Seilheimer and Schachner, 1988; Brummendorf et al., 1989; Johnson et al., 1989; Furley et al., 1990). Each of these molecules is expressed in the developing peripheral and/or central nervous systems, although L1, F11, and TAG-1 tend to be more restricted to axon bundles (Daniloff et al., 1986; Hatta and Takeichi, 1986; Lemmon and McLoon, 1986; Sanes et al., 1986; Hatta et al., 1987; Rathjen et al., 1987a; Dodd et al., 1988; Ranscht, 1988; Furley et al., 1990). Cadherins, NCAM, and L1 all exhibit homophilic binding (eg. N-cadherin to N-cadherin), and L1 also appears to bind an unidentified heterologous ligand

(Rutishauser et al., 1982; Nagafuchi et al., 1987; Grumet and Edelman, 1988; Matsunaga et al., 1988a; Lemmon et al., 1989). N-cadherin is important for ciliary ganglion (CG) neurite outgrowth on astrocytes, Schwann cells, and myotubes while NCAM mediates CG neurite outgrowth on muscle cells only, suggesting that the role of NCAM in CG growth cone motility depends on the particular cellular substrate contacted (Bixby et al., 1987; Tomaselli et al., 1988b; Bixby et al., 1988). L1 has been implicated in neurite outgrowth by a variety of neuronal types on Schwann cells, Muller glia, and on the surfaces of other axons (Chang et al., 1987; Bixby et al., 1988; Drazba and Lemmon, 1990). L1, F11 and an additional CAM, neurofascin, have also been implicated in growth along axonal surfaces and in axon fasciculation (Chang et al., 1987; Rathjen et al., 1987b). The argument that CAMs directly affect growth cone motility is strengthened by the demonstration that purified N-cadherin, L1, and TAG-1 molecules as well as a potentially novel CAM, p84, act as potent substrates for neurite outgrowth in vitro (Lagenaur and Lemmon, 1987; Bixby and Zhang, 1990; Chuang and Lagenaur, 1990; Furley et al., 1990). In addition, the cytoplasmic domains of cadherins and NCAM have been shown to interact with specific cytosolic proteins (Pollerberg et al., 1987; Ozawa et al., 1989).

The overwhelming conclusion of this brief summary is that specific neuronal receptors and their ligands can be identified and shown to mediate neurite outgrowth in vitro. From current knowledge of the mechanics of growth cone motility, we can say that each of the receptors must be active at the growth cone and must, at the very least, serve to affix the growth cone to its substrate. In fact, most of these receptors were initially isolated for their ability to promote the adhesion of whole cells to each other and to ECM substrates long before their roles in promoting growth cone motility were appreciated (Rutishauser 1976; Grunwald et al., 1982; Neff et al., 1982; Yoshida and Takeichi, 1982; Grumet and Edelman, 1984). In promoting neurite outgrowth, is their function merely to mediate the attachment of

the growth cone to the substrate? Or must additional signals be transduced across the membrane?

What is *adhesion*? To *adhere* means "to hold fast or stick by as if by gluing, suction, grasping or fusing" (Webster's New Collegiate Dictionary, 1973). This is obviously not a biochemical definition, but aptly describes the image invoked when cell adhesion is discussed by scientists. The word adhesion in biology is often abused because of this image, and because it has never been conclusively defined by biologists. J.P. Trinkaus (1984) confronted this quandry by observing:

> Since measurement is the basis of precise definition, the measurement of adhesiveness of cells is basic to a definition of cell adhesion. . . The confused state of the art calls for a strictly operational approach. . . In operational terms, adhesiveness might mean many things: the extent and the closeness of the contacts between cells, the breaking strength of contacts (per cell or per area of contact), the work of formation of adhesive bonds, and many others. . . Moreover, all of these properties could vary independently of each other.

A theoretical treatment of the physical chemistry of cell adhesion substantiates this assessment by showing that the instantaneous force of adhesion is a function of the total energy of the chemical bonds between the cell and the substrate divided by the distance between their two surfaces (Bell, 1978). In this discussion, cell adhesion generally reflects the breaking strength of contacts, since each of the above receptors have been shown to maintain cell-cell or cell-substratum sticking under mechanically disruptive conditions that dissociate cells when the mechanism has been neutralized, usually with function-blocking antibodies. However, the phenomenon of cell adhesion is also likely to entail complex subcellular events that do not necessarily reflect receptor-ligand binding in the "adhesion assay". For example, the strength of cell adhesion to protein substrates increases over time and requires energy and actin polymerization (DeGeorge et al., 1985; Lotz et al., 1989). The initial binding of the cell to the substrate has sometimes been called "attachment", and the subsequent strengthening event(s) has been called "adhesion".

Thus, adhesion can mean much more than the simple, biochemical binding of receptor to ligand, making the term *cell adhesion molecule* as ambiguous as possible.

Letourneau (1975) first described the preference of growing neurites for more rather than less adhesive surfaces. However, the point that stickiness is not penultimate to the growth cone was most elegantly made in an experiment by Gundersen (1987) in which neurons were cultured on a collagen substratum that had been dotted with laminin. Neurites grew on the collagen background, but once they had encountered the laminin dot, they grew exclusively within the boundaries of the dot. The force of growth cone attachment to either substrate was measured and was found to be greater on collagen than on laminin. Thus, growth cones preferred to grow on laminin to which they adhered less tightly, suggesting that laminin receptors transduce growth signals to the growth cone in addition to mediating attachment. Bixby (1989) has provided evidence that laminin-stimulated neurite outgrowth depends on the activity of protein kinase C. In addition, genetic evidence implicates an interaction between the Abelson tyrosine kinase with fasciclin I, a neural cell adhesion molecule in *Drosophila*, in growth cone guidance (Elkins et al., 1990a and b). These observations suggest that receptors that promote growth cone motility also modulate intracellular signalling mechanisms. In addition, heterogeneities in the linkage of each receptor to the cytoskeleton might also provide a basis for differential signalling across the growth cone's plasma membrane. These factors are likely to be critical to the activity of neurite outgrowth-promoting receptors in vivo where multiple adhesive systems are likely to be engaged simultaneously.

Evidence for Receptor-Mediated Growth Cone Guidance

There are several types of evidence supporting the view that growth cone guidance and, therefore, pathfinding are receptor-mediated events. One is based on observations of the specificity of growth cone behavior within pathways in cases where the molecules are unknown. Another relies on the identification, where possible, of specific molecules that influence aspects of the pathfinding process. The theme that the growth cones stick and pull continues from the last section, but we now ask about the role of receptors in the directionality of growth. The fusion of these concepts is nicely brought out by Bray (1979) who, speculating on pathfinding mechanisms, wrote:

> Probably the simplest way would be to test the potential contacts mechanically: to apply weight to them just as a mountaineer would test a hand-hold. If the adhesion is sufficient, then the growth cone can contract against it and advance in that direction. If the hold is not secure then the leading edge of the growth cone will be retracted.

It is important to emphasize that although nervous systems are touted for the reproducibility and accuracy of their cellular connections, a certain amount of environmental sampling takes place during pathfinding such that the growth cone, like the mountaineer, makes numerous transient contacts in vivo often leading the axon along routes that are not necessarily the shortest way to their destination (cf. Cajal, 1929; Harris et al., 1987; Thanos and Bonhoeffer, 1987; Nakamura and O'Leary, 1989). The observation that growing axon populations follow some pathways rather than others led to the elaboration of the "blueprint hypothesis" (Singer et al., 1979) and the "substrate pathways hypothesis" (Katz and Lasek, 1979). Both predict the existence of specific cues along preformed anatomical substrates as mechanisms for axonal guidance. The latter deemphasizes the action of soluble factors in determining the trajectories of growth, relying more heavily on the distribution of fixed cues (Katz and Lasek, 1979).

One recurrent suggestion, that channels of extracellular space guide axons, is an historical remnant. W. His, believing that young axons would take the path of

least resistance, was the first to propose that cell-poor spaces within the tissue would be most permissive to axon growth. The notion that spaces themselves guide axon growth is excluded from consideration today, knowing (as Harrison suggested in 1935) that growth cones exert tension on the neurite (cf. Lamoureux et al., 1989). Nevertheless, claims of "non-specific" growth or guidance by extracellular spaces appear in the recent literature (eg., Silver and Sidman, 1980; Krayanek and Goldberg, 1981; Nordlander and Singer, 1982). Any role of these spaces in guidance must be a product of the desirability of the edge of the space (eg., glial endfeet) which must provide the actual cues for growth. However, it does not seem necessary to invoke a role for preformed channels of space, since directed cell movements such as the invasion of cancer cells or the infiltration of lymphocytes into tissues occur by specific chemical means and against the odds of anatomical barriers such as endothelium (cf. Trinkaus, 1984).

From studies of simple nervous systems, such as those of the grasshopper, fruit fly and zebrafish, much evidence has accrued in support of the proposal that specific environmental cues, such as a particular cell or axon fascicle, are required for growth cones to navigate unerringly to their target. These nervous systems are "simpler" than those of higher vertebrates, because they contain comparatively small numbers of cells that can be recognized individually and manipulated experimentally. In the grasshopper and zebrafish central nervous systems, growth cones reproducibly fasciculate with identified bundles of axons (cf. Raper et al., 1983). When these bundles are removed by the laser ablation of cell bodies, growth cones that normally would follow them are left "stranded" and do not reach their targets (Raper, et al., 1984; Kuwada, 1986). In combination with the discovery that such pathways are distinguished by their expression of particular antigenic determinants, this observation led to the "labeled pathways hypothesis" which is reminiscent of Sperry's chemoaffinity hypothesis in that it calls for the activity of

highly pathway-specific molecules to guide small sets of appropriately responsive neurons (Goodman et al., 1984). Subsequent studies of the antigens that label pathways in the insects have uncovered a set of glycoproteins that often bear striking homology with vertebrate neurite outgrowth-promoting molecules, such as NCAM and L1 (Harrelson and Goodman, 1988; Bieber et al., 1989). Two molecules, fasciclins I and III, bear little significant amino acid sequence homology to any known protein but have been shown to mediate cell-cell adhesion in vitro (Snow et al., 1989; Elkins et al., 1990a). In flies transheterozygous for mutations in the fasciclin I and Abelson tyrosine kinase genes, defects in growth cone guidance are detected (Elkins et al., 1990b). Nevertheless, it has been disappointing that even in these genetically manipulable systems, convincing roles for the activity of single identified molecules in pathfinding have not been established. This has led to the modification of the labeled pathways hypothesis from its most stringent interpretation to one that relies on the activity of combinations of molecules. Ironically, this principle was originally voiced to explain growth cone behavior in vertebrates (cf. Bixby et al., 1987; Rathjen et al., 1987a). On the other hand, the the consequences of models deriven from advances made in insects and vertabrates encourage the belief that we are studying the right molecules if not in the right way.

In the grasshopper peripheral nervous system, no specific molecules have been identified. However, a series of elegant and relentless experiments by Bentley and colleagues support the mountaineer's view of pathfinding based on differential growth cone "affinities" for cues in the environment, including guidepost cells, the basal lamina and segment boundaries (Bentley and Caudy, 1983; Caudy and Bentley, 1986; Condic and Bentley, 1989). The word affinity seems to have been chosen in an effort to avoid "adhesion", yet it is clear that the pioneer growth cone sticks to the guiding elements in the pathway, since removal of the basement membrane often causes the neuronal cell body to become dislodged from the

epithelium and to spring forward towards the growth cone (Condic and Bentley, 1989). Furthermore, cytochalasin treatment disrupts pathfinding in the embryo, consistent with the view (above) that the actin-rich filopodia and lamellopodia provide the driving force for directed growth cone motility (Bentley and Toroian-Raymond, 1986). Finally, dissociated afferent neurons selectively aggregate and fasciculate with one another, suggesting that neuron-neuron cues in the afferent pathway are based on specific adhesive mechanisms (Condic, et al., 1989). These results argue that growth cones move from cue to cue within the afferent pathway to the CNS and that molecular recognition events must underly pathfinding in this simpler system.

Interestingly, Bentley's concept of affinity was based in part on the morphologies of growth cones as they encounter pathfinding cues. Prior to firm contact with guidepost cells and at segment boundaries, growth cones are highly complex with a profusion of filopodia extending in virtually all directions (cf. Caudy and Bentley, 1986). Upon full contact with the cue, growth cone structure becomes more compact. Similar observations have been made in the vertebrate nervous system where growth cones are found to have larger lamellopodia and more filopodia at choice points in their pathways (Tosney and Landmesser, 1985c; Bovolenta and Mason, 1987; Harris et al., 1987). The signals associated with these morphological changes are not well understood, but presumably reflect the activity of receptors on the growth cone.

The best evidence for receptor-mediated pathfinding comes from studies in the chick limb where the innervation of specific muscle masses by motor nerve roots has been carefully followed and manipulated. Preferred substrates for motorneurons appear to be the surfaces of other growing axons until the nerve undergoes reductive branching, sending individual growth cones to contact the surface of the muscle (Tosney and Landmesser, 1985a, b, and c; Dahm and

Landmesser, 1988). NCAM, L1, and laminin are all expressed in the limb at appropriate developmental stages, but none delineate the pathway in advance (Rogers et al., 1986; Sanes et al., 1986; Tosney et al., 1986). However, when delivered during embryogenesis, antibodies against NCAM and L1 have significant effects on the development of innervation patterns. Anti-NCAM decreases the number of branches made while anti-L1 increases branching (Landmesser et al., 1988). This is consistent with the view that NCAM mediates growth cone/muscle interactions, whereas L1 is important in axon growth on axons, suggesting that the balance of cues ought ultimately to determine the path of each axon. Most importantly, these experiments show that both NCAM and L1 satisfy the two criteria for molecules that regulate growth cone motility and guidance and are, therefore, bona fide pathfinding molecules. NCAM has also been shown to play a role in the guidance of retinal ganglion cell axons to their central targets in the frog and chicken (Fraser et al., 1984; 1988; Thanos et al., 1984).

The fact that L1 and NCAM distribution in the limb does not delineate the paths later taken by neurons led Landmesser (1988) to propose that two kinds of pathfinding molecules exist: those that provide generally permissive cues (eg. an eight-lane highway) and those that provide highly specific cues (the fast lane). Candidates for the first class abound, but we are at a loss to find members of the second class. Although antigens expressed on some subsets of axons have been identified (eg. TAG-1 in the spinal cord; Dodd et al., 1988), there is no direct evidence that they function in pathfinding.

Another possibility is that the growth cone integrates a number of environmental cues to determine its net rate and direction of growth. Each cue individually might be expressed apparently diffusely, but the overlap of molecules at a given point together with the particular combination of receptors expressed by a given growth cone would be significant parameters in the equation. The growth

cone probably has access to an array of different molecules displayed on the surfaces of other axons, glial cells, basal laminae and in the extracellular space and perhaps a dearth of molecules in cell-poor spaces and on fibroblasts. The role of inhibitory molecules in guiding axon growth is also emerging in a number of systems (cf. Cox et al., 1990; Davies et al., 1990; Raper and Kapfhammer, 1990). Thus, the arguments that pathfinding must be receptor-mediated are reasonably convincing, but it is not clear that distinct molecules must be invoked to specify each pathway individually. Certainly, the fact that the neurite outgrowth-promoting receptors listed in Table 1.1 are broadly expressed and active on many neuronal populations supports the idea that pathfinding represents a series of choices based on the integrated activities of a number of receptors rather than the strict preference of one growth cone for one particular ligand.

One of the obstacles to making an assessment of molecules and pathfinding is the difficulty of the experiments required. The use of antibodies in vivo is troublesome, since (i) the delivery systems that have been useful in some scenarios are not applicable to all, (ii) it is difficult to assign direct neuronal effects in a complex tissue whose integrity may also depend on the activity of the antigen, and (iii) the ability of the antibody to penetrate tissues is always a concern. One alternative is the device of in vitro paradigms that approximate in vivo situations. The use of cryostat sections has been informative in studies of growth in response to peripheral nerve and muscle tissue cues (Covault et al., 1987; Sandrock and Matthew, 1987b; Carbonetto et al., 1987), but this method has not been exploited to its fullest potential. Other methods have permitted the biochemical characterization of factors that influence the directionality of growth (cf. Bonhoeffer and Huf, 1980; 1982; Walter et al., 1987; 1990). With the advent of techniques that permit the monitoring of individual moving growth cones in vivo (cf. Harris et al., 1987) and molecular genetic methods for altering neuronal expression patterns (eg. Holt et al.,

1990), the appreciation of the roles of molecules in genuine pathfinding may be imminent.

The Embryonic Chicken Retina as a Model System

Much is known about the development of the retina and the optic pathway, as it has been the focus of pathfinding studies since the days of Cajal. Many of the advances in growth cone guidance and pathfinding have exploited this system, and testable models exist for the specification of the retino-tectal projection. In addition, several apparently mundane factors have made the embryonic retina a convenient system. The retina is an easily accessible part of the central nervous system, containing millions of cells even at early stages in development. Thus, retinal neurons are amenable to experiments requiring large numbers of cells, such as quantitative attachment assays and biochemical studies. Further, retinal neurons prefer to grow in serum-free medium and in the absence of neurotrophic factors (D.E. Hall, unpublished observations), simplifying considerably experiments that examine the specific interaction of these neurons with defined substrates.

The focus of this thesis is to ask about the potential of certain molecules to promote adhesion and neurite outgrowth by developing retinal neurons in vitro. Experiments have been designed to test reasonable possibilities: either the receptors and/or ligands were known to be expressed in the retina and/or the optic pathway, or there was an experimentally-based suspicion that this would be the case. Such correlations were made when possible. The approach has been primarily immunological, relying on the use of function-blocking antibodies against previously identified neuronal cell surface glycoproteins to perturb adhesion and neurite outgrowth in response to either purified protein substrates or astroglial cell surfaces. In addition, the isolation of two new monoclonal antibodies with these activities and the characterization of their antigens are described. This work is no

substitute for experiments in vivo but has helped to pinpoint important molecules that will be the subjects of future study.

Table 1.1 Molecules that promote neurite outgrowth: Receptors and their ligands.

Receptor	extracellular ligand	intracellular ligand
β_1 Integrins:		talin, a-actinin
$\alpha_1\beta_1$	laminin, collagen	
$\alpha_2\beta_1$	laminin, collagen	
$\alpha_3\beta_1$	laminin, fibronectin, collagen	
$\alpha_4\beta_1$	fibronectin, VCAM	
$\alpha_5\beta_1$	fibronectin	
$\alpha_6\beta_1$	laminin	
$\alpha_7\beta_1$	laminin	
$\alpha_V\beta_1$	fibronectin, vitronectin	
CAMs:		
N-cadherin	N-cadherin	catenins?
NCAM	NCAM, HeSPGs?	fodrin
L1	L1	?
	other	?
MAG	?	?
F11	?	?
TAG-1	?	?
Neurofascin	?	?
p84	?	?

CHAPTER TWO

Embryonic Neural Retinal Cell Response to Extracellular Matrix Proteins: Developmental Changes and Effects of the CSAT Antibody

Abbreviations used in this chapter: BSA, bovine serum albumin; CMF-PBS, calcium and magnesium free phosphate buffered saline; E, embryonic day; EDTA, ethylenediaminetetraacetic acid; MIg, mouse immunoglobulin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; N-CAM, neural cell adhesion molecule; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SUMMARY

Cell attachment and neurite outgrowth by embryonic neural retinal cells were measured in separate quantitative assays to define differences in substrate preference and to demonstrate developmentally regulated changes in cellular response to different extracellular matrix glycoproteins. Cells attached to laminin, fibronectin and collagen IV in a concentration-dependent fashion, though fibronectin was less effective for attachment than the other two substrates. Neurite outgrowth was much more extensive on laminin than on fibronectin or collagen IV. These results suggest that different substrates have distinct effects on neuronal differentiation.

Neural retinal cell attachment and neurite outgrowth were inhibited on all three substrates by two antibodies, CSAT and JG22, which recognize a cell surface glycoprotein complex known to be required for cell interactions with several extracellular matrix constituents. In addition, retinal cells grew neurites on substrates coated with the CSAT antibodies. Both results suggest that cell surface molecules recognized by this antibody are directly involved in cell attachment and neurite extension.

Neural retinal cells from embryos of different ages varied in their capacity to interact with extracellular matrix substrates. Cells of all ages, embryonic day 6 (E6) to E12, attached to collagen IV and CSAT antibody substrates. In contrast, cell attachment to laminin and fibronectin diminished with increasing embryonic age. Age-dependent differences were found in the profile of proteins precipitated by the CSAT antibody, raising the possibility that modifications of these proteins are responsible for the dramatic changes in substrate preference of retinal cells between E6 and E12.

INTRODUCTION

Neuronal interaction with defined tissue culture substrates has been a useful experimental paradigm leading to the elucidation of some of the molecular requirements for cell attachment and neurite outgrowth as well as cellular events leading to neurite extension. Defined factors from both conditioned medium (Collins, 1978; Adler et al., 1981; Coughlin et al., 1981; Lander et al., 1982) and extracellular matrices (Lander et al., 1982) stimulate neurite extension by cultured neurons. The extracellular matrix protein laminin is active alone and appears to account for the neurite outgrowth promoting activity of conditioned medium (Lander et al., 1985; Davis et al., 1984). Substrate attached laminin stimulates neurite outgrowth by diverse neuronal cell types from both the peripheral and central nervous systems (Baron von Evercooren et al., 1982; Manthorpe et al., 1983; Smalheiser et al., 1984; Rogers et al., 1983; Lander et al., 1983; Unsicker et al., 1985; Faivre-Bauman et al., 1984; Adler et al., 1985). Fibronectin is also an effective substrate for neurite outgrowth by peripheral neurons (Baron von Evercooren et al., 1982; Carbonetto et al., 1983; Rogers et al., 1983), but less so for central neurons (Carbonetto et al., 1983; Rogers et al., 1983). Collagens I and IV support neurite extension under some conditions (Carbonetto et al., 1983; Vlodavsky et al., 1982).

Embryonic neural retina, the subject of the present study, is a useful system for studying neuronal interactions with extracellular matrices. Previous work implicates the extracellular matrix in regulating the development of retinal ganglion cells. In the goldfish and chick retinae, axons are initiated in proximity to a basement membrane, the inner limiting membrane (Easter et al., 1984; McLoon, 1984). Within the eye, axons grow on three substrates: existing axons, Muller glial endfeet and the inner limiting membrane. Enzymatic removal of the inner limiting membrane during the period of axon outgrowth results in disorganized growth of axons but does not affect preexisting axons (Halfter and Deiss, 1984). Therefore, axonal growth within the eye seems to

depend upon cell interactions with molecules present in the basement membrane. Laminin is present in the inner limiting membrane of developing chick (McLoon, 1984; Adler et al., 1985) and rat retina, as well as transiently in rat optic stalk (personal communication, Dr. S. McLoon, University of Minnesota). As laminin stimulates neurite outgrowth by neural retinal cells in vitro (Rogers et al., 1983; Adler et al., 1985) and is appropriately localized in vivo, it is likely to influence axon initiation and growth in the developing retina.

Recently, progress has been made in identifying neuronal surface molecules which may mediate retinal cell interactions with basement membranes. The monoclonal antibody, T61, disorganizes and inhibits axonal growth in retinal explants (Halfter and Deiss, 1986). Two other monoclonal antibodies, CSAT and JG22, which compete with each other, perturb cell/matrix interactions in a variety of cell types (Neff et al., 1982; Horwitz et al., 1985; Decker et al., 1984; Bozyczko and Horwitz, 1986; Chen et al., 1985b; Greve and Gottlieb, 1982). The glycoproteins recognized by these antibodies include a fibronectin receptor (Akiyama et al., 1986; Horwitz et al., 1985) and have some properties of a laminin receptor (Horwitz et al., 1985). The cDNA-derived protein sequence of the subunits of the fibronectin receptor indicate that they contain hydrophobic transmembrane segments (Tamkum et al., 1986; Argraves et al., 1986). The CSAT proteins colocalize and interact with microfilament associated proteins (Damsky et al., 1985; Horwitz et al., 1986). Therefore, these proteins have appropriate properties to mediate interactions between the matrix and cytoskeleton. In our experiments, the CSAT and JG22 antibodies were used to determine whether neural retinal cells possess similar molecules that mediate neural responses to purified extracellular matrix molecules.

MATERIALS AND METHODS

Materials: Linbro 96 well flat bottom tissue culture plates were purchased from Flow Laboratories, McLean, Va. EN^{3}HANCE was purchased from New England Nuclear, Boston, Ma. Na^{125}I and L-{35S} methionine were obtained from Amersham, Arlington Hts., IL. Iodogen was from Pierce Chemicals, Rockford, IL. Protein A Sepharose CL-4B and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Fertile White Leghorn Chicks were from Feather Hill Farm, Petaluma, Ca. and mice were from Simonson Laboratories, Gilroy, Ca. Mouse Immunoglobulin (IgG) was purchased from Cappell, Malvern, Pa. Affinity purified polyclonal antibodies to murine collagen IV were obtained from Dr. H. Furthmayr, Yale University, New Haven, Conn. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma #M2138) and other chemicals were purchased from Sigma Chemical Company, St. Louis, Mo.

Protein Purification and Antibody Preparation: Murine collagen IV and laminin were purified from the Engelbreth-Holm-Swarm sarcoma by published procedures (Timpl et al., 1982). Human cell fibronectin was a gift from Dr. S. Fisher, University of California, San Francisco. Proteins were radiolabelled by the Iodogen method (Fraker and Speck, 1978). Hybridoma cells secreting CSAT and JG22 antibodies were gifts from Dr. A.F. Horwitz, University of Pennsylvania, Philadelphia, Pa., and Dr. D. Gottlieb, Washington University, St. Louis, Mo., respectively, and were grown as described (Neff et al., 1982). To prepare large amounts of antibodies, hybridoma cells were injected into pristane primed 8-week old female Balb C mice. After ascites fluid was collected, the CSAT antibodies were purified by Protein A-Sepharose CL-4B chromatography (Neff et al., 1982). Rabbit anti-neural cell adhesion molecule (anti-N-CAM) antibodies and IgG were prepared by Dr. J. Bixby (Bixby and Reichardt, 1987).

Neurite Outgrowth and Attachment Assays: Both the neurite outgrowth and attachment assays had four major steps: substrate preparation, neural retinal cell preparation, plating and incubation of neural retinal cells, and analysis for either attachment or neurite outgrowth. Lower cell densities and longer incubation times were used on the plates to be analyzed for neurite outgrowth. Otherwise, the two assays used identical protocols.

To prepare substrates, sterile 96 well plates were coated with 100 µl per well of laminin, collagen IV or antibody diluted in calcium and magnesium-free phosphate buffered saline (CMF-PBS; 200 mg/l KCl, 200 mg/l K_2SO_4, 8·0 g/l NaCl and 2.16 g/l $Na_2HPO_4 \cdot 7H_2O$, pH 7.36-7.45) to the concentration indicated in the results. Fibronectin was applied in 100 µl per well of sterile 0.1 M cyclohexylaminopropane sulfonic acid (CAPS) buffer, pH 9.0. Plates were incubated with proteins overnight at room temperature. After rinsing 3 to 5 times with sterile CMF-PBS, the wells were blocked by incubation with 1 % BSA for at least 2 hours at room temperature. Plates were again rinsed 3 - 5 times with sterile CMF-PBS and 100 µl of culture medium was added to each well. At this time antibodies were added to the wells and plates were stored in the incubator, at 37°C and 5% CO_2, until the cells were ready, ca 1 hr.

Retinae were dissected from embryonic chickens in CMF-PBS containing 0.6% glucose and 100 U/ml penicillin and streptomycin. The retinal cells were dissociated by trypsinization in 0.03% trypsin for 10 minutes in CMF-PBS followed by trituration. Dissociated cells were collected by centrifugation and resuspended for preplating in F12 nutrient mixture with insulin (5 µg/ml), selenium (30 nM), iron saturated ovotransferrin (25 µg/ml), and 100 U/ml penicillin and streptomycin (Bottenstein et al., 1980). Cells were preplated on tissue culture plates for 45 minutes at 37°C in a 5% CO_2 atmosphere to remove non-neuronal cells. At the end of the preplating step, the supernatants containing neuronal cells were harvested and the cell number was adjusted for cell

attachment and neurite outgrowth experiments. For attachment assays, the neural retinal cells were suspended at approximately 5-10 x 10^5 cells per ml. For the neurite outgrowth assay, cells were used at 10^4 cells per ml. Lower initial plating densities were used for the neurite outgrowth assay since it was difficult to assess neurite number in wells with more than about a thousand cells. For both assays, 100 µl of cells per well were added to the previously prepared plates and these were centrifuged in a Beckman TJ-6 centrifuge at 48 x g for 5 min. This centrifugation step greatly increased the reproducibility of the attachment assay since it removed the variability inherent in settling of cells onto the culture plate surface.

Attachment assay plates were analyzed after incubation at 37°C in a 5% CO_2 atmosphere for 1.5 hrs. Unattached cells were removed by the brisk addition of warm F12 nutrient mixture to each well followed by gentle vacuum suction with a narrow bore pipet. The cells were fixed overnight in the cold in 2% glutaraldehyde in F12 nutrient mixture and stained 2 to 4 hrs. the following morning by the addition of trypan blue to a final concentration of 0.08% (Koda et al., 1986). After thorough rinsing, the A_{690} of individual wells was measured on a microtiter plate reader (Flow Laboratories, McLean, Va.). In early experiments, we determined that cell number was proportional to A_{690}. All plates used for cell attachment assay included wells coated with 1 mg/ml poly-D-lysine as a positive control and wells coated with 1% BSA for negative controls. Early experiments showed that 100% of the plated cells attached to poly-D-lysine and less than 5% attached to BSA. The percentage of cells attached to experimental wells was calculated from the following formula:

A_{690} (experimental well) - A_{690} (BSA-coated well) /
A_{690} (Poly-D-lysine-coated well) - A_{690} (BSA-coated well)

Plates containing cells to be analyzed for neurite outgrowth were grown for 16-24 hours at 37°C in a 5% CO_2 atmosphere. Cells were fixed with 2% glutaraldehyde as described above and examined in the microscope. Neurite outgrowth was quantified by determining the percentage of cells bearing neurites > 2 cell diameters in length (Lander et al., 1982). The viability of cells cultured for longer periods was measured by the cell survival assay of Manthorpe et al (1986). Briefly, 10 µl of MTT solution was added to each culture one hour before fixation. Viable cells take up and convert MTT to a visible blue formazan product.

Immunoprecipitation with CSAT Antibody: Chick embryo fibroblasts prepared as previously described by Rein and Rudin (1968) and retinal cells prepared as in the previous section, were metabolically labelled by incubation overnight at 37°C in a 5% CO_2 atmosphere with 60 µCi/ml [^{35}S]-methionine in methionine-deficient F12 medium. After labelling, the fibroblasts and neural retinal cells were incubated for 10 minutes at 37°C in CMF-PBS with 5 mM EDTA and then removed from the dish by gentle pipetting. Cells were resuspended in 0.5 ml Hepes-buffered F12 nutrient mixture, and 40 µg of antibody (CSAT or mouse immunoglobulin, MIg) was added. After one hour incubation at room temperature, cells were extracted in Extraction Buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 0.5% Triton-X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4) for 30 minutes at 4°C with occasional vortexing. The extracts were spun at 10,000 x g for 1 min. to pellet insoluble material. Supernatants were cleared twice by incubation with Sepharose 4B for 1 hr. at 4°C followed by centrifugation for 1 min (134 x g in a Beckman TJ6). Immune complexes were adsorbed by mixing with Protein A-Sepharose CL-4B (75 µl of packed beads per tube). After 1 hr., Sepharose beads were sedimented at 134 x g (Beckman TJ6 for 10 sec.) and washed 4 times with 10 ml Extraction Buffer. Pellets were resuspended in 100 µl electrophoresis sample buffer and boiled for SDS-PAGE analysis (Laemmli, 1970).

7.5% gels were run under reducing (with beta-mercaptoethanol) or non-reducing conditions, treated with EN^{3}HANCE, dried, and exposed to Kodak X Omat R film.

RESULTS

Embryonic Day 6 Neural Retinal Cells Attach to Extracellular Matrix Glycoprotein Substrates.

Neural retinal cell attachment to different constituents of the extracellular matrix was measured using a quantitative attachment assay (see Methods). Results in Figure 2.1 show that E6 chick neural retinal cells attached to laminin, fibronectin and collagen IV in a concentration dependent fashion. At optimal concentrations of collagen IV and laminin, attachment was as efficient as to poly-D-lysine. At the highest concentrations of fibronectin tested, attachment was about 70% as efficient as to poly-D-lysine. Plates coated with 1-2 μg/ml of collagen IV and 5-10 μg/ml of laminin yielded maximal attachment. Half-maximal attachment was seen with ca 0.5 μg/ml collagen IV and ca 2.5 μg/ml laminin. Fibronectin coated substrates did not support attachment of 100% of the cells plated, even at coating concentrations as high as 300 μg/ml; half maximal attachment required ca 40 μg/ml of fibronectin.

To determine whether observed differences in cell attachment were attributable to variations in protein coating efficiency, known amounts of protein were labelled with ^{125}I and used to measure the efficiency of protein attachment to the plastic substrate (Figure 2.2). Collagen IV was adsorbed about 2-fold more efficiently than fibronectin, which in turn was somewhat more efficiently adsorbed than laminin. Data from Figures 2.1 and 2.2 show that maximal cell attachment was seen with about 7 ng of laminin and 12 ng of collagen IV adsorbed to an area of about 0.28 cm^2 (bottom of well).

The specificity of neural retinal cell interactions with laminin and collagen IV coated substrates was demonstrated using affinity purified anti-collagen IV antibody and anti-laminin antiserum (Figure 2.3). Cells were plated on collagen IV or laminin in the presence or absence of antibodies to laminin, collagen IV or nonimmune serum. Results in Figure 2.3 show that affinity purified anti-collagen IV antibodies inhibited

cell attachment to collagen IV substrates but not to laminin substrates. Conversely, anti-laminin antibodies inhibited neural retinal cell attachment to laminin but not collagen IV substrates. These results provide strong evidence that attachment to laminin and collagen IV involve specific and distinct interactions.

Embryonic Neural Retinal Cells Extend Neurites in Culture when Plated on Purified Extracellular Matrix Proteins.

When neural retinal cells were incubated for longer periods on extracellular matrix protein substrates, some cells extended neurites (Figure 2.4). On laminin, E6 embryonic neural retinal cells attached quickly and assumed a characteristic polygonal shape (Figure 2.4A). These cells developed neurites as early as a few hours after plating, and gave rise to extensive and highly branched networks of neurites by 16 hours (Figure 2.4A). In comparison, the neurites observed on collagen IV or fibronectin were shorter, straighter and had fewer branches (Figure 2.4B,C). Neurite bundles on collagen IV or fibronectin substrates also appeared much thicker and more highly fasciculated than those on laminin.

Neurite outgrowth by neural retinal cells on each of the three substrates was quantified (Figure 2.5). At optimal concentrations of laminin as many as 70% of the neural retinal cells developed neurites longer than two cell diameters by 16 - 24 hours. In contrast, on poly-D-lysine substrates, only 1-5% of the neurons developed neurites. Neurite outgrowth on laminin was concentration dependent (Figure 2.5). Maximal neurite outgrowth required coating plates with 5-10 μg/ml laminin; half-maximal neurite outgrowth required about 2-3 μg/ml laminin. As indicated above, similar concentrations of laminin were required for maximal and half-maximal attachment of neural retinal cells. Only a low level of neurite outgrowth was seen on fibronectin or collagen IV substrates (Figure 2.5). Additionally, neurite outgrowth on collagen IV did not appear to be dependent on the amount of adsorbed ligand. On collagen IV, 20-30%

of the cells developed neurites at concentrations within the range that was effective for cell attachment (compare to Figure 2.1). When the effects of different concentrations of fibronectin were measured, the lowest concentration of fibronectin used in the attachment assay (Figure 2.1) induced neurite outgrowth by about 15% of the cells. Increasing concentrations of fibronectin induced a higher percentage of neurons to extend neurites. At 300 μg/ml of fibronectin, a concentration which permitted attachment of 70% of the cells in the attachment assay, about 40% of the cells extended neurites. While neither collagen IV nor fibronectin promoted profuse neurite outgrowth, both were more effective than poly-D-lysine.

Effects of the CSAT and JG22 Antibodies on Retinal Cell Attachment and Neurite Outgrowth.

CSAT and JG22 antibodies have been shown to bind an apparent complex of three glycoproteins which includes a functional fibronectin receptor (Horwitz et al., 1985; Greve and Gottlieb, 1982; Akiyama et al., 1986) and has some of the properties expected of a laminin receptor (Horwitz et al., 1985). We used these antibodies to determine whether glycoproteins recognized by the CSAT antibody were important in mediating the response of neural retinal cells to extracellular matrix glycoproteins. Attachment of neural retinal cells to laminin, fibronectin and collagen IV was inhibited in the presence of CSAT antibody (Figure 2.6). However, the CSAT antibody had no effect on cell attachment to poly-D-lysine or concanavalin A (data not shown), a finding which suggests that the antibodies interfered specifically with attachment to the protein ligands. Cells were also unable to initiate neurites when cultured overnight in the presence of CSAT antibodies (Figure 2.6). The dramatic inhibitory effects of the CSAT antibody on retinal cell attachment and neurite outgrowth were also seen using the JG22 antibody, which binds the same glycoprotein triplet (Greve and Gottlieb, 1982; Chapman, 1984) (data not shown).

If, as indicated above, the proteins bound by the CSAT and JG22 antibodies are present on the surfaces of neural retinal cells, these antibodies should promote cell attachment when adsorbed onto tissue culture plates (MacLeish et al., 1983; Chen et al 1985b). Results in Figure 2.7 show that plates coated with CSAT (JG22) antibody or anti-N-CAM antibody promoted cell attachment while those coated with antibodies specific for the precursor to β-NGF did not. Substrates of CSAT and JG22, but not anti-N-CAM IgG, supported neurite outgrowth (Figure 2.7, data for CSAT not shown). Neurite outgrowth on CSAT antibody-coated substrates was concentration dependent (data not shown). Cells plated on CSAT or JG22 antibodies were similar in appearance to those plated on laminin. They attached readily, assumed a polygonal shape and extended neurites within several hours after plating. The neurites were frequently long and often individual, rather than fasciculated as on collagen IV or fibronectin. The results in this section provide two types of evidence that neural retinal cells possess surface proteins bound by CSAT and JG22 antibodies. First, CSAT and JG22 antibodies disrupted both cell attachment and neurite outgrowth on extracellular matrix substrates. Second, the antibodies supported cell attachment and neurite outgrowth when adsorbed onto tissue culture substrates.

Developmental Changes in the Responsiveness of Neural Retinal Cells to Extracellular Matrix Proteins.

Neural retinal cells undergo many developmental changes between E6 and E12 in vivo. It seemed possible that these would be accompanied by changes in their properties in vitro. To examine this, neural retinal cells were isolated from embryos of different ages and assayed for the capacity to respond to laminin, fibronectin, collagen IV and CSAT antibody substrates. Attachment and neurite outgrowth were measured in separate quantitative assays. The results, presented in Figure 2.8, show that adsorbed CSAT antibodies were effective at promoting the attachment of neural retinal cells,

regardless of the age of the embryo (E6 through E12). Collagen IV was also an effective substrate for cell attachment throughout this period. In contrast, while 60% of the E6 cells plated attached to fibronectin and 90% to laminin, the ability of cells to attach to these substrates declined sharply with age and was absent by Ell (Figure 2.8). Interaction with laminin was examined in greater detail by determining the concentration dependence of attachment for cells of different ages (Figure 2.9). Half-maximal attachment of E7 cells required only 1-2 μg/ml of laminin, while 2-fold and 3-fold more laminin was needed to obtain equivalent attachment with E8 and E9 cells, respectively. With cells from E12 embryos, even 40-fold higher concentrations of laminin failed to promote equivalent attachment (Figure 2.9).

The morphology of cells grown overnight on extracellular matrix glycoproteins also varied with embryonic age. Within several hours after plating, E6 embryonic retinal cells assumed a characteristic polygonal shape, and extended neurites on laminin (Figure 2.10). Although retinal cells of all ages have a tendency to aggregate, most E6 cells attached directly to the substrate. In contrast, many of the cells from E8 embryos adhered to each other in aggregates which were loosely attached to the substrate. Some E8 cells appeared well attached and had a polygonal shape similar to the E6 cells. In general, polygonal cells appeared to be well attached to the culture substrate, and these cells extended neurites (Figure 2.10B). Few E12 cells attached to the laminin substrate, even after 24 hours of culture. A trend toward more cell aggregation and less substrate interaction was also observed for cells plated on either fibronectin or collagen IV, after 24 hours of culture (data not shown). Aggregates of unattached E12 cells that had been in culture for 16 to 24 hours were viable since they transported MTT, indicating mitochondrial function (data not shown, Manthorpe et al., 1986).

Quantitation of neurite outgrowth revealed a decrease on all of the substrates tested with increasing embryonic age (Figure 2.8). Neurite outgrowth on fibronectin and collagen IV, while never comparable to that observed on laminin, decreased to even

lower levels with increasing embryonic age. This was also true of neurite outgrowth on CSAT antibody substrates (Figure 2.8), which declined at approximately the same rate as the response to laminin. Neurite outgrowth on laminin-coated substrates remained concentration dependent through E9 (Figure 2.9). In contrast to cell attachment, there was no clear shift in the amount of laminin required to induce a half maximal neurite outgrowth response.

Characterization of Surface Proteins on Neural Retinal Cells That Are Precipitated by CSAT Antibodies.

Alterations in the composition or activity of molecules recognized by the CSAT antibody may be related to the observed changes in neuronal response to extracellular matrix proteins. In order to investigate this possibility, we examined the proteins bound by CSAT antibodies on retinal cells of different ages. Dissociated neural retinal cells were obtained from embryos of different ages, preplated to remove non-neuronal cells, and metabolically labelled. Chick embryo fibroblasts were used for comparison to previously described proteins precipitated by CSAT and JG22 antibodies (Horwitz et al., 1984; Hasegawa et al., 1985; Knudsen et al., 1985).

We found several small but reproducible differences between retinal cells from different aged embryos. In agreement with previous reports (Horwitz, et al. 1985; Hasegawa et al., 1985; Knudsen et al., 1985), surface proteins precipitated by CSAT antibodies from chick embryo fibroblasts consisted of two major bands, of Mr 160 K and Mr 130-135 K, when fractionated under reducing conditions (Figure 2.11). Proteins obtained by immunoprecipitation from embryonic neural retinal cells exhibited two major bands and one or more minor bands. The higher molecular weight retinal band was less diffuse than the fibroblast protein of similar molecular weight and migrated in a slightly different position (Figure 2.11). The lower molecular weight retinal protein band was diffuse, but unlike that obtained from chick embryo fibroblasts, resolved into

several components as shown by the densitometric scans. E6 retinal cells had a distinct band of Mr 115 K, verified by densitometry, that was not present in fibroblast samples and was less prominent in both the E8 or E11 retinal samples (Figure 2.11).

Developmental differences in the retinal CSAT-precipitable proteins were more obvious upon non-reducing SDS-PAGE, where the fibroblast CSAT glycoproteins were separated into four distinct components of Mr 145 K, 135 K, 125 K and 110 K. Again, the bands present in the retinal samples were similar, but not identical to those of fibroblasts (Figure 2.12). A band of Mr 145 K was present both in chick embryo fibroblast and in retinal immunoprecipitates of each age. A middle band, of Mr 135 K, was present in all four samples, but migrated as a doublet in the fibroblast and E11 retinal samples (Figure 2.12). Densitometry confirmed that this band migrated as a doublet. While a diffuse protein band of Mr 120 K was also present in all four samples, E6 retinal cells had an additional band of Mr 110 K that was less prominent in the other samples and could be seen as a shoulder on the densitometric scan. The nature of the relationship between these developmentally regulated structural differences and variation in response to extracellular matrix molecules clearly warrants further investigation.

DISCUSSION

Neural Retinal Cells Respond Differently To Three Matrix Glycoproteins.

The use of separate quantitative assays for cell attachment and neurite outgrowth has shown that neural retinal cells respond specifically but differently to collagen IV, laminin and fibronectin. Attachment to all three substrates was concentration dependent (Figure 2.1). Laminin and collagen IV were both effective substrates for cell attachment. Maximal attachment was seen with about 7 ng of laminin and 12 ng of collagen IV to an area of about 0.28 cm^2 (bottom of well). Both of these were more effective substrates than fibronectin. Even when about 90 ng of adsorbed fibronectin was tested, only about 70% of the plated cells attached. Thus, retinal cells have a relatively low affinity for fibronectin, which corroborates previous work (Rogers et al., 1983).

Stimulation of neurite outgrowth by laminin was also distinctive in several ways. First, a high proportion of cells, 70%, initiated neurites on laminin substrates, compared to less than 40% on either fibronectin or collagen IV. Second, the morphology of neurites on laminin substrates was unique among the substrates tested. Neurites on laminin were long, branched and usually not faciculated. In contrast, the neurites observed on either fibronectin or collagen IV were frequently short, straight and fasciculated. A differential response to laminin and fibronectin substrates has previously been reported for retinal and spinal cord neurons. Peripheral neurons, in contrast, respond well to both fibronectin and laminin (Rogers et al., 1983).

Differences in the substrate requirements for cell attachment and neurite outgrowth suggest that while adherence to a substrate is a prerequisite for neurite outgrowth, it is not sufficient. Neurons need an appropriately adhesive substrate for neurite initiation and elongation (Letourneau, 1975, 1982). However, the present study and other recent work using defined substrates indicate that adhesivity may not be as

tightly coupled to neurite outgrowth as previously thought (see also Adler et al., 1985). In our experiments, less than 10% of the cells plated on poly-D-lysine, a very adhesive substrate, had neurites when examined after 16 to 24 hours in culture. Collagen IV, also a very effective substrate for neural retinal cell attachment, supported only modest neurite outgrowth. In contrast, fibronectin, which was notably less effective than collagen IV for cell attachment, proved to be a somewhat better substrate for neurite outgrowth. Only laminin, among the extracellular matrix glycoproteins tested, promoted both efficient cell attachment and vigorous neurite outgrowth. Thus, while neural retinal cells are able to attach to a variety of substrates, different substrates have distinct consequences on neuronal differentiation.

The Role of Molecules Recognized by the CSAT and JG22 Antibodies In Mediating Neural Retinal Response to Matrix Molecules.

The CSAT (and JG22) monoclonal antibodies have been previously shown to disrupt cell interactions with many extracellular matrix macromolecules including fibronectin and laminin (Greve and Gottlieb, 1982; Neff et al., 1982; Akiyama et al., 1986; Horwitz et al., 1985). Interactions of dorsal root ganglion neurons with laminin, fibronectin and collagen I substrates are perturbed by the CSAT antibody (Bozyczko & Horwitz, 1986). In our experiments, low levels of CSAT antibodies (1 - 10 µg/ml) were shown to inhibit neural retinal cell attachment to laminin, fibronectin and collagen IV but not to poly-D-lysine or concanavalin A. The lack of effect on attachment to poly-D-lysine indicates that these antibodies act to inhibit specific interactions with extracellular matrix proteins, and not in a general way to impede cell attachment. Furthermore, CSAT and JG22 antibodies do not inhibit neuron attachment to monolayers of non-neuronal cells (Cohen et al., 1986; Tomaselli et al., 1986) or to mixed substrates of laminin and anti-N-CAM antibodies (Tomaselli et al., 1986). The

inhibition by CSAT antibodies of neural retinal cell interaction with collagen IV substrates extends the range of cell/substrate interactions perturbed by these antibodies.

Neural retinal cells maintained overnight in the presence of CSAT antibody were unable to initiate neurites. Since these cells were not attached to the substrate, the experiments do not address whether CSAT antibody interfered directly with neurite initiation and extension. Two types of evidence suggest that molecules recognized by the CSAT antibody have a direct role in neurite extension and adhesion of the neurite and growth cone. Neurons cultured on CSAT antibody substrates grew neurites, suggesting that under these artificial conditions at least, cell surface molecules recognized by the CSAT antibody acted to anchor not only the cell body but also the growth cone. In addition, ciliary neurons cultured in the presence of CSAT antibody on mixed substrates of either laminin and anti-N-CAM IgG or laminin and poly-D-lysine were found to attach, but not to initiate neurites (Tomaselli et al., 1986). Therefore, under conditions where the cells were attached, CSAT antibody still inhibited neurite extension. The presence of CSAT immunostaining on neurites and growth cones as well as on cell bodies (Bozyczko and Horwitz, 1986) and the ability of CSAT antibody to detach existing neurites from laminin substrates (Bozyczko and Horwitz, 1986) are also consistent with a direct role of molecules recognized by CSAT in neurite extension.

Substrate-attached CSAT antibodies mimicked, to a degree, the effects of laminin on neural retinal cells (Figure 2.4). Neural retinal cells attached to plates coated with anti-N-CAM or CSAT antibody but not to those coated with control antibodies. However, CSAT and JG22, but not anti-N-CAM antibodies, promoted vigorous neurite outgrowth when used as substrates (Figure 2.4). These results are similar, in some respects, to effects on neural crest cell and fibroblast adhesion to JG22-coated substrates (Duband et al., 1986; Chen et al., 1985b). Substrate adsorbed JG22 antibodies promoted attachment and spreading of both cell types. (Chen et al., 1985b). Although neural crest cells normally migrate on fibronectin, they failed to migrate on JG22 antibody

substrates, indicating that the antibodies did not completely mimic the effects of fibronectin (Duband et al., 1986). In our experiments, laminin was a more effective substrate for neurite extension than the CSAT antibody.

Retinal Cell Response to Matrix Molecules is Developmentally Regulated.

Results in this paper show that neuronal responsiveness to matrix macromolecules changes with age. Collagen IV was an effective substrate for cell attachment throughout the embryonic period examined, E6 through E12. Substrate adsorbed CSAT antibody remained able to promote cell attachment by E12 neural retinal cells, demonstrating the persistence of surface molecules recognized by the CSAT antibody. The continued presence of molecules recognized by the CSAT antibody on E12 cells was confirmed by immunoprecipitation, discussed below. In contrast, neuronal responsiveness to laminin and fibronectin declines with age. Attachment to fibronectin, already comparatively low on E6, declines over the next three days and is absent by E9. Attachment to laminin remains high between E6 to E9 but declines by E11. Examination of dose response curves shows that cells become progressively less responsive to laminin between E6 and E9 and that most become completely unresponsive by E11. This suggests that neuronal receptors for laminin and fibronectin, but not those for collagen IV, are down-regulated during this time.

The ability of neural retinal cells to initiate neurites on extracellular matrix protein substrates also varied markedly with the age of the neurons. Laminin, CSAT antibody, and to a lesser extent fibronectin and collagen were all effective substrates for neurite initiation by E6 neurons. Neurite outgrowth on fibronectin was decreased by E8. The ability to extend neurites persisted longer for cells plated on either laminin or CSAT antibody but was absent by E12. Since E12 neural retinal cells are able to extend neurites when cultured on monolayers of astrocytes, the lack of neurite extension on matrix substrates does not reflect a loss of the ability to extend neurites (Cohen et al.,

1986; K. Tomaselli, unpublished results). Developmental changes in the responsiveness of ciliary ganglion neurons to substrates coated with heart cell conditioned medium have also been shown (Collins and Lee, 1982). More recently, ciliary neurons have been shown to initiate neurites on monolayers of non-neuronal cells at a time when the cells did not respond to laminin coated substrates (K. Tomaselli, unpublished results). Since the stimulation of neurite outgrowth by non-neuronal cells is not inhibited by the CSAT and JG22 antibodies (Cohen et al., 1986; Tomaselli et al., 1986), these interactions must be mediated by cell surface molecules other than those recognized by these antibodies.

The continued presence of molecules recognized by the CSAT antibody, demonstrated by attachment to CSAT antibody substrates and by immunoprecipitation, indicates that lack of cell attachment to laminin and fibronectin, and absence of neurite outgrowth by E12 cells cannot be attributed to the loss of such molecules from the cell surface. The proteins recognized by CSAT antibody on retinal neurons appear similar, but not identical, to those on fibroblasts (Figures 2.11 and 2.12). There were, however, discernible differences in the pattern of proteins precipitated by the CSAT antibody from E6, E8 and E12 retinal cells. Two differences in the pattern of proteins from cells of different ages were seen consistently. The E6 sample had an additional component, of Mr 110-115 K, that was not present in either fibroblasts or in the other retinal samples. On non-reducing gels, the 135 K component migrated as a single band in the E6 and E8 retinal samples, but as a doublet in the E12 retinal and fibroblast samples. In contrast, the affinity purified antigen from sensory neurons may lack the high moleculer weight band seen on non-reducing gels (Bozyczko and Horwitz, 1986). The significance of these differences is not known. Several lines of evidence from our and other laboratories suggest the possibility that molecules recognized by the CSAT antibody may belong to a family of receptors, of closely related structure, but with distinct ligand specificities (Leptin, 1986; Horwitz et al., 1985; Pytela et al., 1985;

Ruoslahti and Pierschbacher, 1986; Pytela et al., 1986). One possibility is that retinal cells of different ages have structurally related but distinct cell surface molecules, all recognized by the CSAT antibody, that mediate interaction with different matrix glycoproteins.

Whether the loss of neuronal responsiveness to laminin and fibronectin reflects the loss of a specific external receptor or of an intracellular coupling event is unknown. It is clear from our data that molecules recognized by the CSAT antibody are present on E11 retinal cells, that these cells retain the ability to attach to collagen IV, and that cell interaction with collagen IV is disrupted by the CSAT antibody. However, whether the subtle differences we observed in the pattern of proteins immunoprecipitated with the CSAT antibody from E6 and the E11 cells are sufficient to explain the different ligand specificities of the cells at these two developmental times is unclear.

Possible Roles for The Extracellular Matrix In Regulating Retinal Neuron Development.

In many developing systems, neuronal growth cones have been found in contact with basal laminae, which may be expected to contain collagen IV and laminin (Raper et al., 1983; Taylor and Roberts, 1983; Easter et al., 1984; Rogers et al., 1986; McLoon, 1984). In the retina, axonal growth cones have extensive contact with the inner limiting membrane and other recently formed axons, suggesting that both axon surface and extracellular matrix constituents may be important in directing growth cone behavior (Easter et al., 1984). Evidence suggests that molecules in the basement membrane stimulate axon initiation and provide directional cues to elongating axons (Halfter & Deiss, 1984; 1986). Our experiments suggest that the interactions with laminin and other basement membrane glycoproteins involve neuronal cell surface molecules recognized by the CSAT and JG22 antibodies.

Changes in the responsiveness of neural retinal cells to extracellular matrix constituents observed in vitro may be related to the in vivo development of retinal ganglion cells. In the chick, retinal ganglion cells are generated between E2.5 and E7 (Kahn, 1973). Because ganglion cell birth rate is exponential, many more cells become postmitotic (Kahn, 1973) and initiate axons toward the end of this period (Goldberg and Coulombre, 1972; Halfter et al., 1983). The growth of the first axons to appear is likely to involve the basement membrane since no other axons are present initially (Halfter et al., 1985). Axon elongation within the eye, occurring for the greatest number of axons around days 6 and 7, is also likely to involve interactions with basement membrane (Halfter and Deiss, 1984), while elongation within the optic nerve and optic tectum may primarily involve interactions with cell surfaces (Thanos and Bonhoeffer, 1983). In this regard, it is particularly interesting that E12 retinal ganglion cells, the majority of which would have axons that had reached the optic tectum, retain the ability to extend axons on astrocyte surfaces but not on extracellular matrix molecules (Cohen et al., 1986). The growth of neurites on monolayers of astrocytes, Schwann cells and skeletal myotubes is not inhibited by the CSAT antibody (Tomaselli et al., 1986). Thus, neurons have multiple receptors that can promote neurite outgrowth on different substrates. Our observations are consistent with the possibility that these receptors are developmentally regulated to ensure that axon growth is appropriately guided at different stages.

Figure 2.1: Embryonic neural retinal cells attach to tissue culture substrates coated with purified extracellular matrix molecules. A 1.5 hour attachment assay was used to measure the attachment of E6 chick neural retinal cells to tissue culture substrates coated with purified laminin, fibronectin or collagen IV as described in Methods. Wells coated with 1 mg/ml poly-D-lysine served as a positive control; attachment to poly-D-lysine was taken as 100% and was used in the calculation of per cent cells attached for the other substrates. Each point is the average of at least three separate determinations. The Figure shows per cent of cells attached vs. coating protein concentration for collagen IV (squares) laminin (circles) and fibronectin (triangles). The error bars show standard error of the mean.

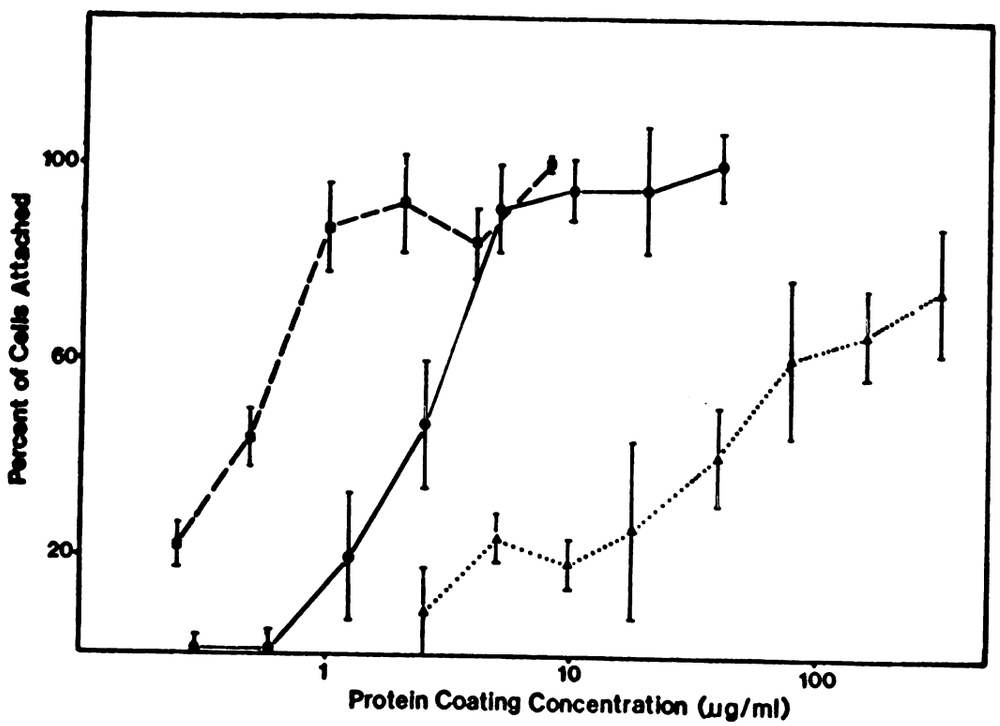

Percent of Cells Attached
100
60
20
1
10
100
Protein Coating Concentration (µg/ml)

Figure 2.2: Quantitation of protein binding to tissue culture plastic wells. Laminin, fibronectin, collagen IV and CSAT antibody, iodinated to high specific activity (see Methods), were applied to tissue culture plastic wells as for attachment and neurite outgrowth assays. Wells were rinsed with CMF-PBS then extracted with NaOH until all radiolabel was removed. Amount of protein bound was calculated from the known specific activity of the labelled proteins. Amount of protein bound (nanograms) vs. amount of protein applied (micrograms) is shown for laminin (open squares), fibronectin (closed squares), collagen IV (open circles) and CSAT antibody (closed circles).

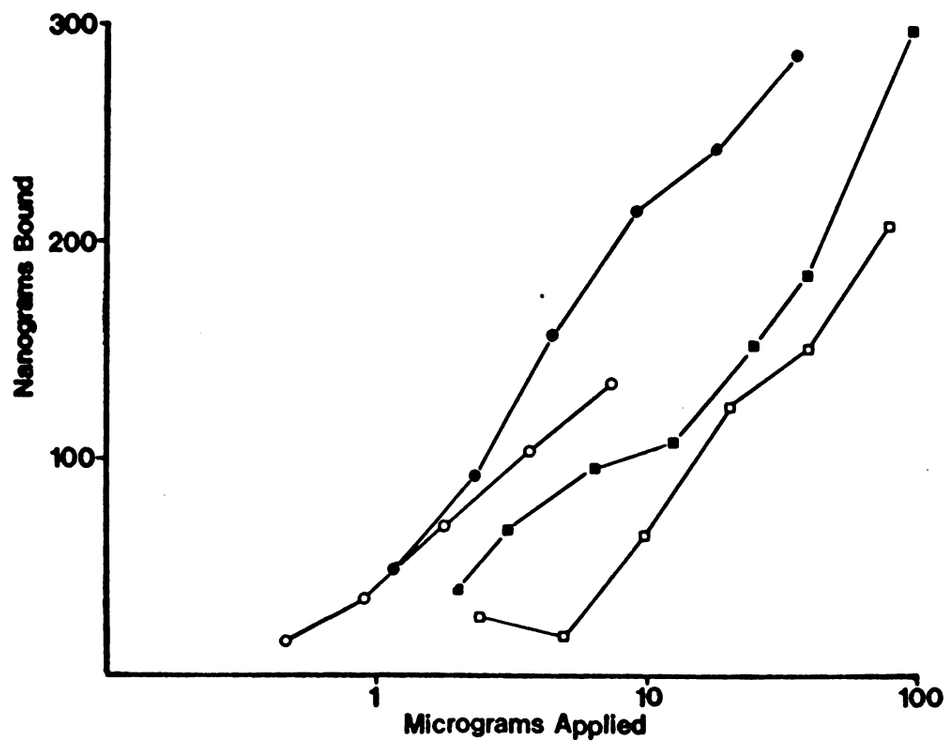

300
200
100
Nanograms Bound
1
10
100
Micrograms Applied

Figure 2.3: Embryonic neural retinal cells attach to laminin and collagen IV in a specific fashion. Day 6 embryonic neural retinal cells were assayed for attachment to protein coated tissue culture wells as described in Methods. Cells were plated onto wells that had been coated with 2 μg/ml collagen IV (Top Panel) or 5 μg/ml laminin (Bottom Panel) without antibodies (A), with preimmune serum (B), with affinity purified anti-collagen IV antibodies (C), or with anti-laminin antiserum (D). Wells coated with poly-D-lysine were used as a positive control in the calculation of per cent of total cells attached. Error bars show standard error of the mean.

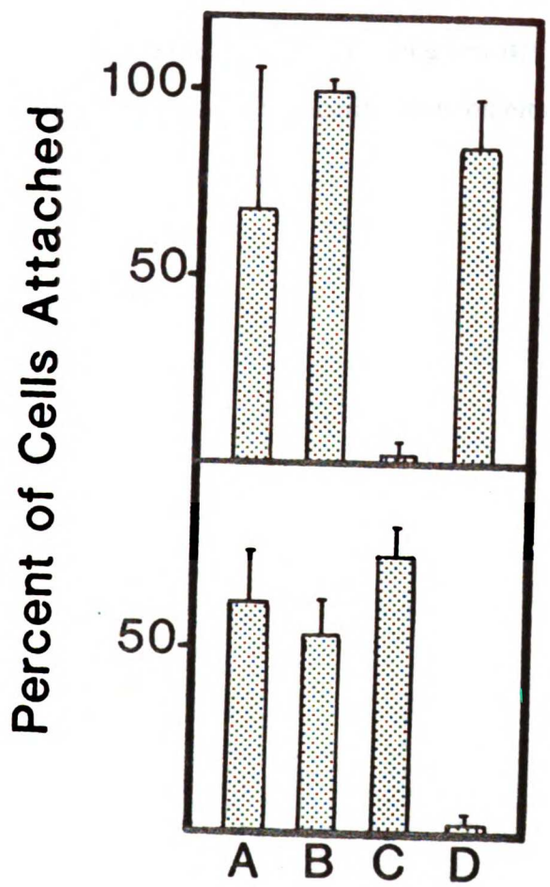

Percent of Cells Attached
100
50
50
A
B
C
D

Figure 2.4: Phase photomicrographs of E6 neural retinal cells plated on extracellular matrix protein substrates. E6 neural retinal cells were plated onto tissue culture plastic wells that had been coated with 5 µg/ml laminin (A), 300 µg/ml fibronectin (B) or 2 µg/ml collagen IV (C). The cells were maintained in culture for 24 hours as for neurite outgrowth assay (see Methods). Cells were fixed with 2% fresh glutaraldehyde in cold PBS then photographed. Compare the abundant and long neurites from cells plated on laminin (A) with the more sparse and shorter neurites seen on either fibronectin (B) or collagen IV (C) substrates. Bar, 20µm.

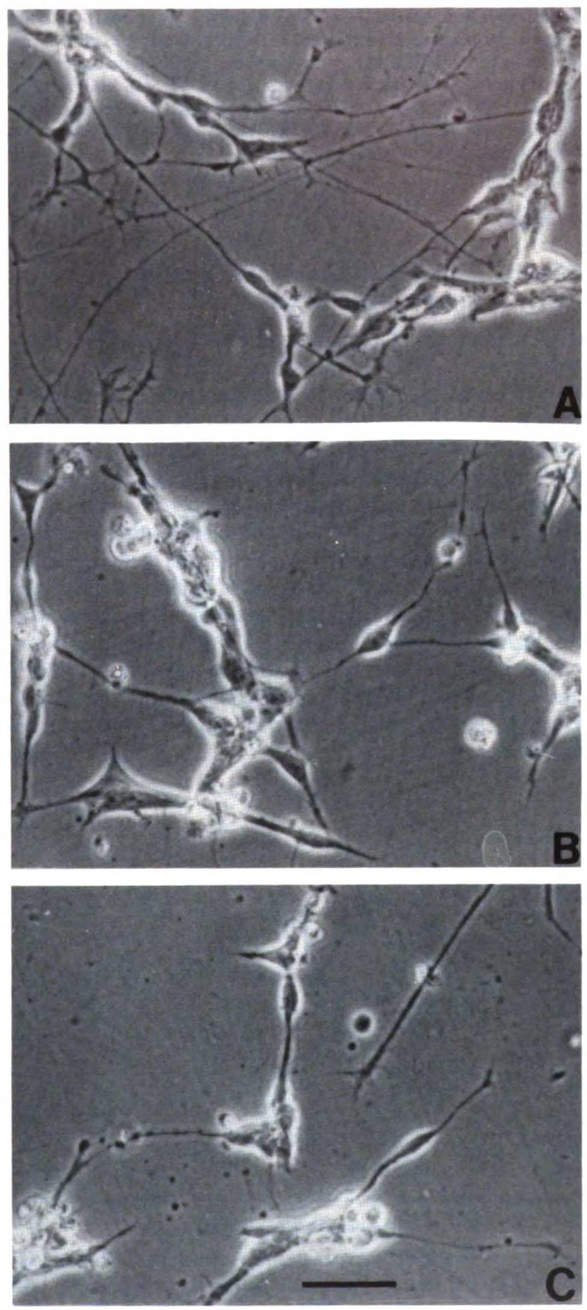

A
B
C

Figure 2.5: Quantitation of neurite outgrowth by embryonic neural retinal cells cultured on laminin (circles), fibronectin (triangles) or collagen IV (squares) substrates. E6 retinal cells were plated on tissue culture wells coated with either laminin, fibronectin or collagen IV and maintained in culture in serum-free medium for 16 to 24 hours. Cells were fixed with 2% glutaraldehyde in PBS and percent of cells bearing neurites was determined as described in Methods. Each point is the average of at least three separate determinations. Percent of cells bearing neurites vs. protein coating concentration is shown, the error bars show standard error of the mean.

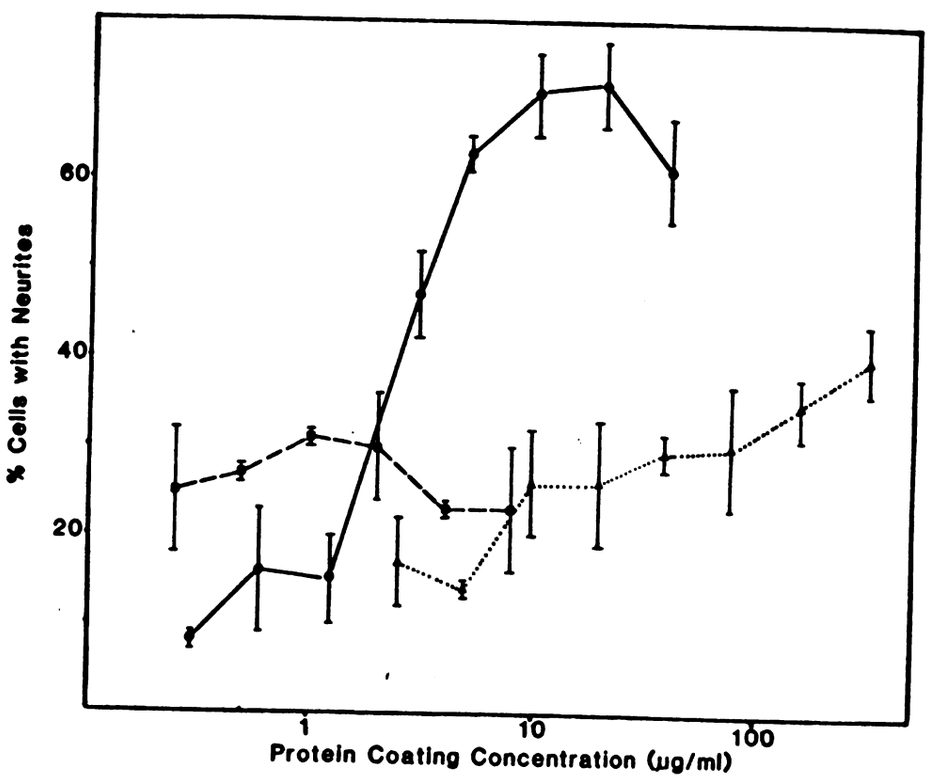

% Cells with Neurites
60
40
20
1
10
100
Protein Coating Concentration (µg/ml)

Figure 2.6: CSAT antibody inhibition of embryonic neural retinal cell attachment and neurite outgrowth on laminin, fibronectin and collagen IV. E6 neural retinal cells plated on wells coated with 5 μg/ml laminin, 300 μg/ml fibronectin or 2 μg/ml collagen IV were assayed separately for attachment and neurite outgrowth as described in Methods. Measurements were done without antibody (open bars), with 10 μg/ml mouse IgG (stippled) or with 10 μg/ml CSAT (black). Attachment to poly-D-lysine in the presence and absence of CSAT antibody is also shown. Error bars show the standard error of the mean.

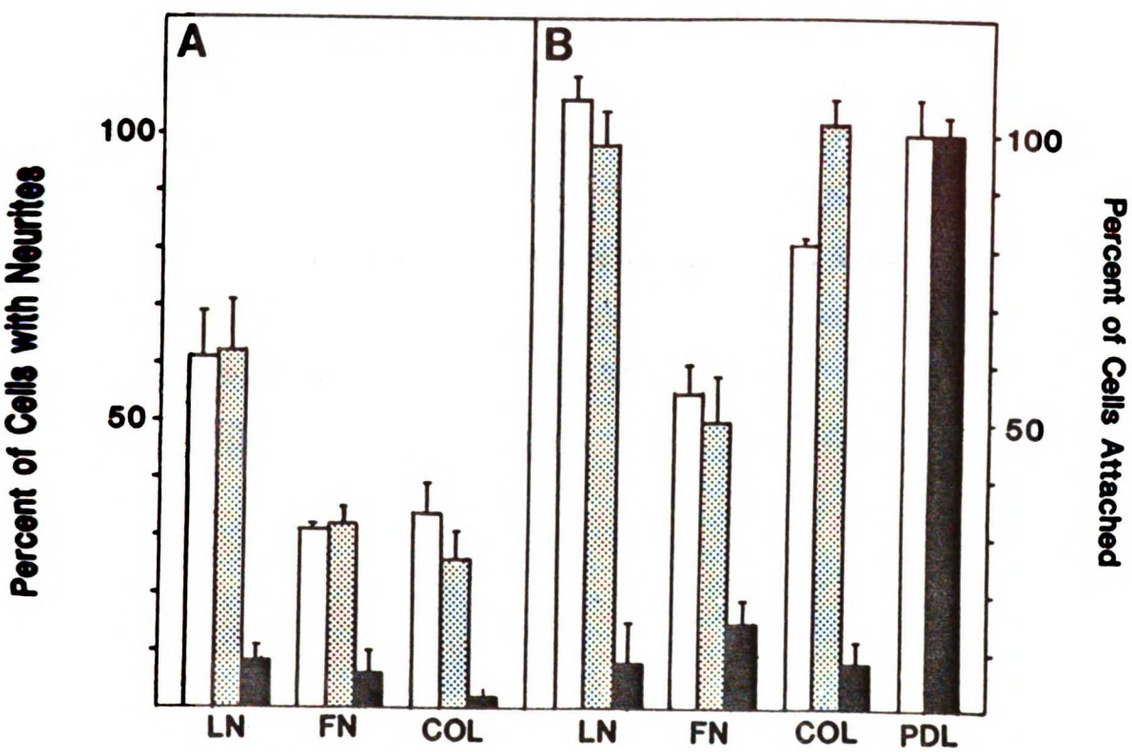

A
B
100
50
100
50
Percent of Cells with Neurites
Percent of Cells Attached
LN
FN
COL
LN
FN
COL
PDL

Figure 2.7: Phase photomicrographs of embryonic neural retinal cells plated on antibody coated substrates; 75 µg/ml JG22 (A), 100 µg/ml Anti-N-CAM (B) or 75µg/ml Anti-P34 (C). E6 neural retinal cells were plated onto wells coated with antibodies and maintained in culture for 16 to 24 hours then fixed with 2% glutaraldehyde and photographed. Polygonal, well attached and spread cells with neurites are seen on JG22 antibody substrates (A). Cells on the anti-N-CAM antibody substrate appear well attached and spread but have few neurites (B). The arrow in B shows a broad lamellopodial region. Loosely attached cell aggregates are observed in cultures on anti-P34 antibody substrates (C). Bar, 26µm.

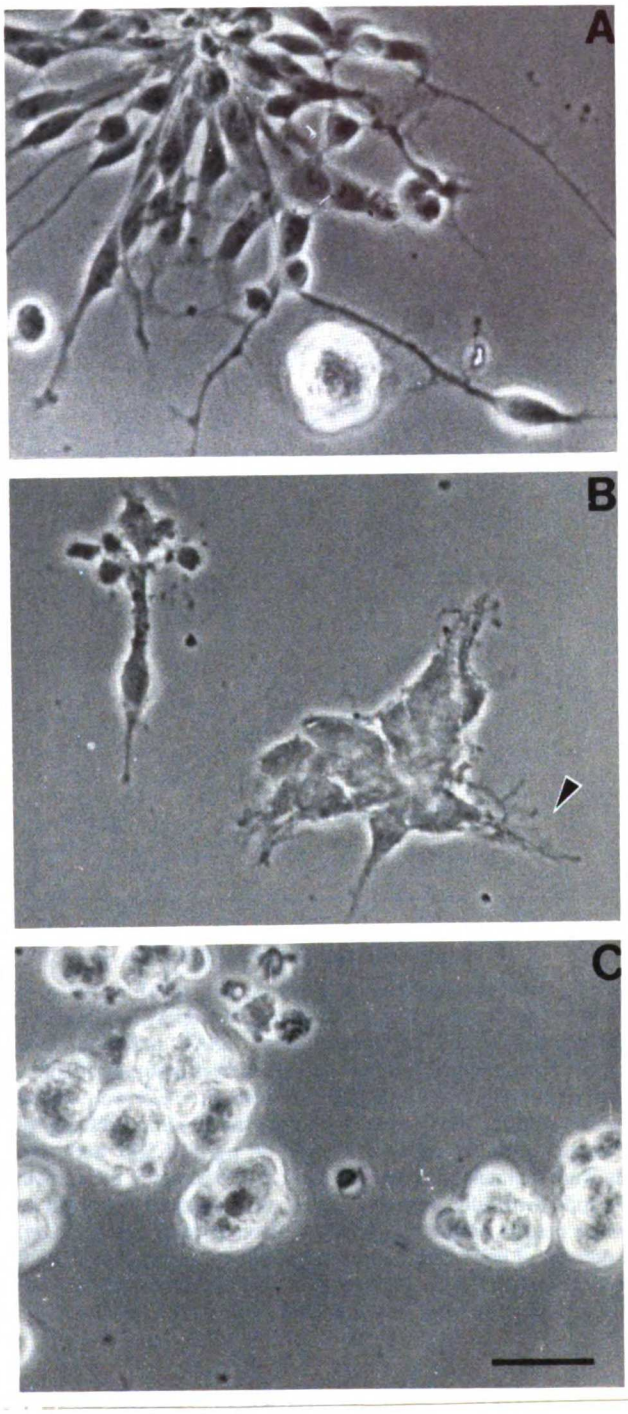

A
B
C

Figure 2.8: Developmental change in neural retinal cell response to laminin (circles), fibronectin (open triangles), collagen IV (closed triangles), and substrate attached CSAT antibody (squares). Neural retinal cells were obtained from embryos of different age as described in Methods. Cells were plated on tissue culture wells coated with 5μg/ml laminin, 300 μg/ml fibronectin, 2 μg/ml collagen IV and 50 μg/ml CSAT antibody. Cells were maintained in culture for 1.5 hours to measure attachment (top panel) and identically prepared plates were cultured overnight to measure neurite outgrowth (bottom pannel). Percent cells attached (top panel) and per cent of cells with neurites (bottom panel) vs. embryonic day are shown. Each point is the average of at least three separate determinations. The error bars show standard error of the mean.

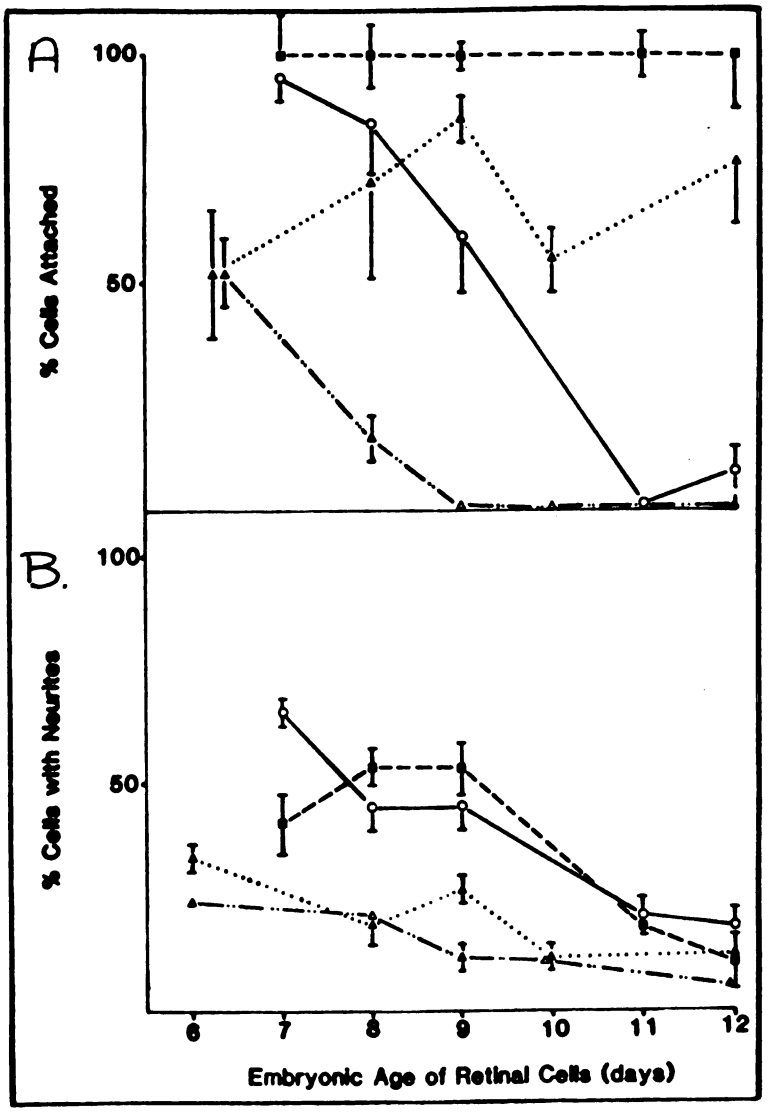

A
100
50
% Cells Attached
B.
100
50
% Cells with Neurites
6
7
8
9
10
11
12
Embryonic Age of Retinal Cells (days)

Figure 2.9: Embryonic neural retinal cells respond differently to substrate attached laminin depending on embryonic age. Attachment and neurite outgrowth were measured separately for neural retinal cells obtained from day 7, 8, 9, 11, and 12 embryos. Cells were obtained from retinae of each age, preplated, then plated onto wells coated with laminin, ranging in concentration from 0.3 μg/ml to 40 μg/ml. In the top panel, percent cells attached vs. laminin concentration is shown for cells from different embryonic days. The bottom panel shows neurite outgrowth for the same period, measured on identical, but different plates. The error bars show standard error of the mean. Embryonic day is noted at the right of the figure next to each curve.

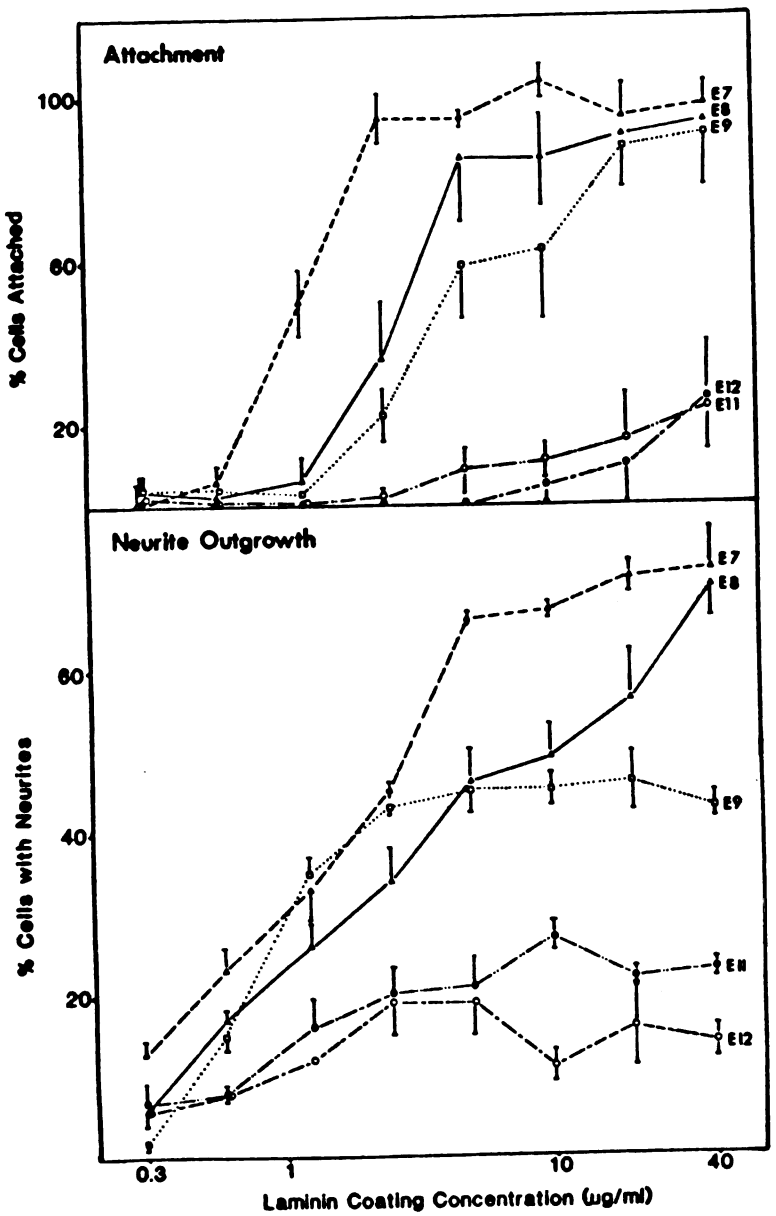

Attachment
% Cells Attached
100
60
20
E7
E8
E9
E12
E11
Neurite Outgrowth
% Cells with Neurites
60
40
20
E7
E8
E9
E11
E12
0.3
1
10
40
Laminin Coating Concentration (ug/ml)

Figure 2.10: Phase photomicrographs of neural retinal cells from embryos of different age plated on laminin coated substrates. Neural retinal cells from E6 (A), E8 (B) or E12 (C) were plated on substrates coated with 5 μg/ml laminin and photographed after 24 hours in culture. E6 neural retinal cells exhibit a well attached, polygonal morphology with many long neurites (A). Some E8 cells appear well attached and have neurites (arrow, B), while others are in large cell aggregates that are primarily out of the plane of focus (star, panel B). E12 neural retinal cells are primarily unattached, in large cell aggregates (arrow, C) with a few attached cells, but no neurites. Bar, 20μm.

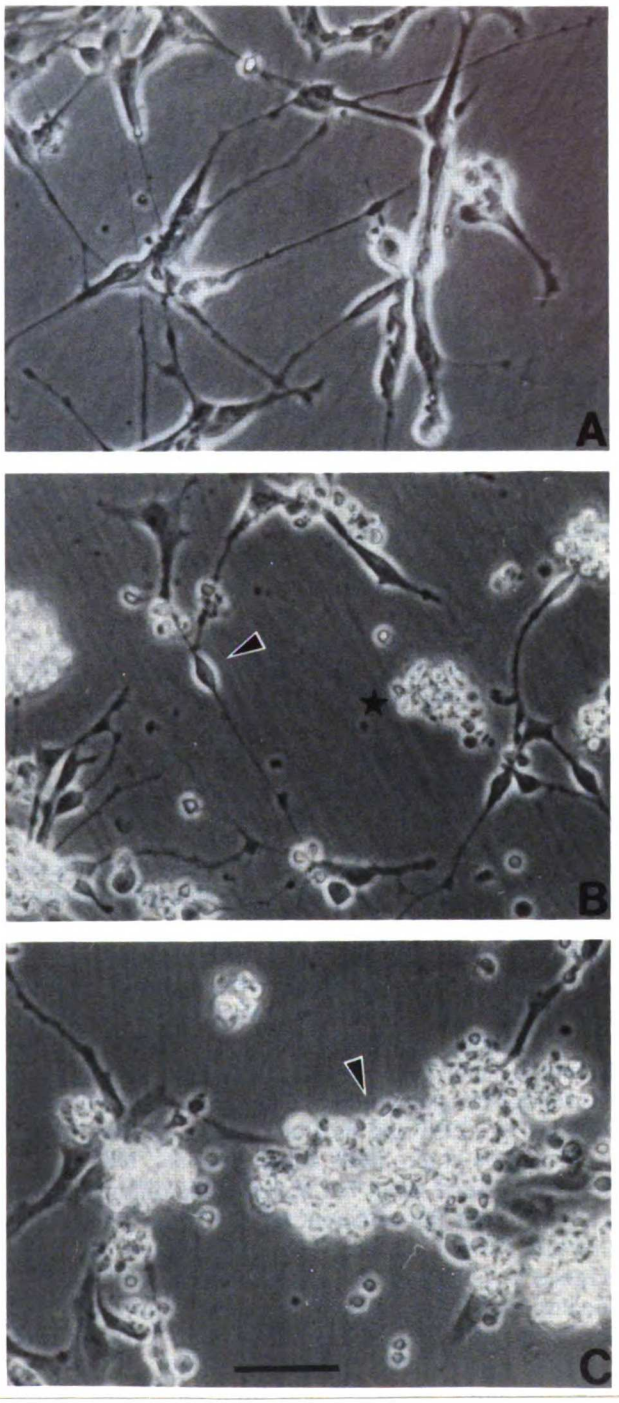

A
B
C

Figure 2.11: Reducing SDS-PAGE analysis of proteins immunoprecipitated with CSAT antibody from chick embryo fibroblasts and embryonic neural retinal cells. The right panel shows a fluorograph of a 7.5% SDS-PAGE run under reducing conditions. Lanes marked "C" were loaded with CSAT immunoprecipitates from chick embryo fibroblasts (CEF) and embryonic neural retinal cells from different days of development; E6, E8 and E11. "M" lanes show non-immune mouse IgG control immunoprecipitates. The left panel shows densitometry of the autoradiograph.

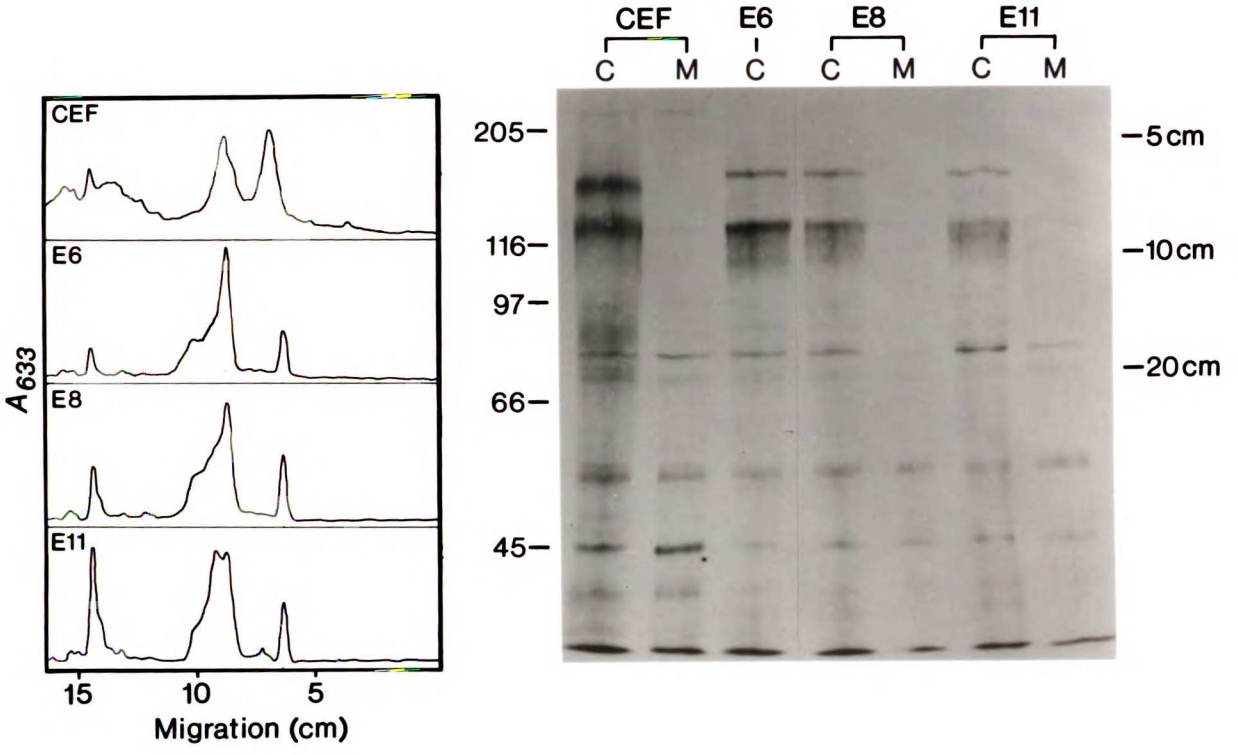
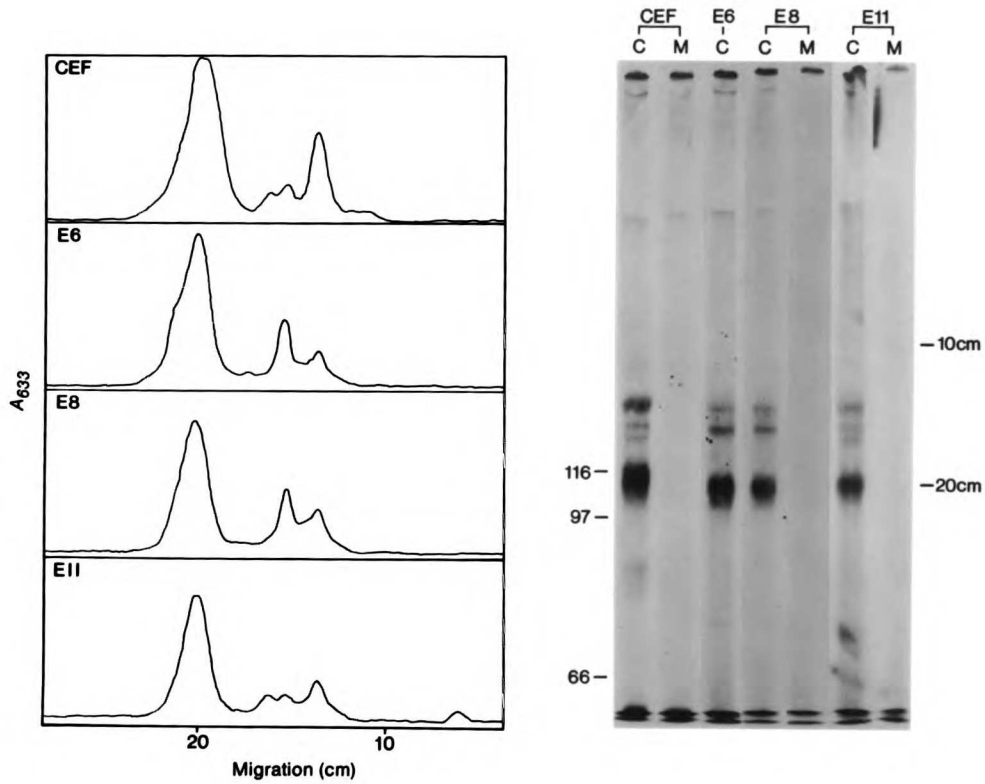

CEF
E6
E8
E11
A_{633}
15
10
5
Migration (cm)
CEF
E6
E8
E11
C
M
C
C
M
C
M
205
116
97
66
45
5cm
10cm
20cm

Figure 2.12: Non-reducing SDS-PAGE analysis of proteins immunoprecipited with CSAT antibody from chick embryo fibroblasts and embryonic neural retinal cells. The right panel shows a fluorograph of a 7.5% SDS-PAGE run under non-reducing conditions. Lanes marked "C" were loaded with CSAT immunoprecipitates from chick embryo fibroblasts (CEF) and embryonic neural retinal cells from E6, E8 and E11. Non-immune mouse IgG precipitates are shown in adjacent lanes (M) for comparison. Densitomety of the autoradiograph is shown in the left panel. The chick embryo fibroblast (CEF) sample and neural retinal cell samples of different ages, (E6, E8 or E11), are indicated.


CEF
E6
E8
E11
A_{633}
20
10
Migration (cm)
CEF
E6
E8
E11
C
M
C
C
M
C
M
116
97
66
10cm
20cm

CHAPTER THREE

N-cadherin, NCAM, and Integrins Promote Retinal Neurite Outgrowth on Astrocytes In Vitro

Abbreviations used in this chapter: E, embryonic day; CAM, cell adhesion molecule; NCAM, neural cell adhesion molecule.

SUMMARY

Retinal ganglion neurons extend axons that grow along astroglial cell surfaces in the developing optic pathway. To identify the molecules that may mediate axon extension in vivo, antibodies to neuronal cell surface proteins were tested for their effects on neurite outgrowth by embryonic chick retinal neurons cultured on astrocyte monolayers. Neurite outgrowth by retinal neurons from embryonic day 7 (E7) and E11 chick embryos depended on the function of a calcium-dependent cell adhesion molecule (N-cadherin) and β_1-class integrin extracellular matrix receptors. The inhibitory effects of either antibody on process extension could not be accounted for by a reduction in the attachment of neurons to astrocytes. The role of a third cell adhesion molecule, NCAM, changed during development. Anti-NCAM had no detectable inhibitory effects on neurite outgrowth by E7 retinal neurons. In contrast, E11 retinal neurite outgrowth was strongly dependent on NCAM function. Thus, N-cadherin, integrins, and NCAM are likely to regulate axon extension in the optic pathway, and their relative importance varies with developmental age.

INTRODUCTION

In the developing primary visual system of vertebrates, the endfeet of neuroepithelial astroglial precursors are prominent components of the routes taken by retinal ganglion cell axons within the eye, optic nerve, and optic tectum (Rager, 1980; Easter et al., 1984; Silver and Rutishauser, 1984; Lemmon, 1985; 1986; Bork et al., 1987). In the retina, growth cones contact the endfeet of radially oriented glia as well as the inner limiting basement membrane as they grow toward the optic stalk (Rager, 1980; Easter et al., 1984). In the optic nerve, small fascicles of axons are separated by glial processes, and their growth cones frequently contact glial surfaces (Rager, 1980; Silver and Rutishauser, 1984; Bovolenta and Mason, 1987). Although the role of glial surfaces in the ordered growth of optic fibers into the tectum is unknown, the optic nerve fiber layer of the embryonic tectum is penetrated by numerous radial glial endfeet (Lemmon, 1985; 1986; Bork et al., 1987). Thus, observations of optic pathway development in vivo suggest that astroglial precursors stimulate and guide the elongation of retinal ganglion cell axons.

Studies of central nervous system development and regeneration have demonstrated that astrocytes provide an effective cellular substrate for axonal elongation (Bohn et al., 1982; Smith et al., 1986). Astrocytes isolated from embryonic or early postnatal brain are good substrates for rapid neurite extension by both central and peripheral neurons in vitro (Noble et al., 1984; Fallon, 1985a & b; Tomaselli et al., 1986). In particular, embryonic chick retinal neurons extend profuse, unfasciculated neurites on astrocyte surfaces, while they do not respond as well to fibroblast monolayers (McCaffery et al., 1984; Fallon, 1985a). Therefore, astrocytes are distinct from other cell types in that they express highly active neurite outgrowth-promoting factors. Retinal neurons also attach and extend neurites on substrates coated with the purified extracellular matrix (ECM) proteins, laminin, fibronectin, and collagen types I and IV (Akers et al., 1981; Adler et al., 1985; Cohen et al., 1986; Hall et al., 1987; K.

Neugebauer, unpublished observations). Laminin, which is the most effective of these ECM proteins in promoting neurite outgrowth by retinal neurons (Hall et al., 1987), is expressed on the surfaces of cultured astrocytes (Liesi et al., 1983). Recent evidence indicates, however, that laminin is not the only neurite outgrowth-promoting factor expressed by astrocytes. First, retinal neurons isolated from embryonic day 11 (E11) chick embryos no longer respond to purified laminin, yet they continue to extend neurites rapidly on astrocyte surfaces (Cohen et al., 1986; Hall et al., 1987). Second, neurite outgrowth by peripheral neurons on astrocytes is only mildly inhibited by a monoclonal antibody (CSAT) that completely inhibits neurite outgrowth on these ECM-derived substrates (Tomaselli et al., 1988b). This antibody recognizes the integrin β_1 subunit shared by a family of ECM receptor heterodimers (see Hynes, 1987 for nomenclature) and blocks neuronal attachment and process outgrowth on laminin, fibronectin, collagens, and native ECMs (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986, 1987a; Cohen et al., 1987; Hall et al., 1987). Thus, cultured astrocytes express neurite-promoting factors that are distinct from these ECM proteins.

Recently, attention has been focused on the role of cell adhesion molecules (CAMs) as cell surface-associated neurite-promoting factors. N-cadherin is a 130 kD cell surface glycoprotein that mediates calcium-dependent adhesion between neural cells (Grunwald et al., 1982; Hatta et al., 1985) and is expressed by both central and peripheral neurons (Hatta et al., 1986; 1987; Crittenden et al., 1988). Neurite outgrowth by peripheral neurons grown on astrocytes depends strongly on the function of N-cadherin (Tomaselli et al., 1988b). N-cadherin also functions in peripheral neurite outgrowth on skeletal myotubes and Schwann cells (Bixby et al., 1987; 1988). The calcium-independent CAM, NCAM, is also widely distributed in the nervous system (cf. Daniloff et al., 1986). Although it promotes neuronal adhesion and process outgrowth on myotubes in vitro (Rutishauser et al., 1983; Bixby and Reichardt, 1987; Bixby et al., 1987), NCAM does not appear to be involved in peripheral neurite

outgrowth on astrocytes or Schwann cells (Tomaselli et al., 1988b; Bixby et al., 1988) or on the surfaces of other peripheral axons (Chang et al., 1987). However, as NCAM is expressed on astrocytes in culture (Noble et al., 1985; Keilhauer et al., 1985), it remains a candidate for mediating interactions that lead to neurite outgrowth by some types of neurons on astrocytes.

As a first step towards defining the molecular mechanisms underlying axon extension in the retinotectal pathway, we have examined embryonic retinal neurite outgrowth on astrocytes in vitro, using antibodies that inhibit the function of N-cadherin, NCAM, and the integrin β_1-class ECM receptor heterodimers. Our results implicate all three of these neuronal receptors in neurite outgrowth by retinal neurons on astrocytes and suggest changes in the relative importance of NCAM function as development proceeds.

MATERIALS AND METHODS

Animals: Fertile White Leghorn chicken eggs were purchased from Feather Hill Farm (Petaluma, CA) and were maintained at 38°C and 95% humidity until use. Mice were from Simonson Laboratories (Gilroy, CA), and newborn Sprague-Dawley rats were from Bantin and Kingman (Fremont, CA).

Chemicals and Reagents: Murine laminin was purified from the Engelbreth-Holm-Swarm sarcoma by Dr. D.E. Hall using published procedures (Timpl et al., 1979). L-(^{35}S)-methionine was from Amersham (Arlington Hts., IL). Aquasol was from New England Nuclear (Boston, MA). Protein A Sepharose CL-4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). DEAE cellulose (DE-52) was from Whatman, Inc. (Clifton, NJ). Pepsin was from Worthington Diagnostics (Freehold, NJ), and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Antibodies: Hybridoma cells secreting the CSAT monoclonal antibody were the generous gift of Dr. A.F. Horwitz, University of Illinois, Urbana, IL and were grown as described (Neff et al., 1982). CSAT recognizes an epitope on the avian integrin β_1 subunit and will henceforth be referred to as anti-integrin β_1 (Buck et al., 1986; see Hynes, 1987 for integrin nomenclature). The 224-1A6-A1 (1A6) hybridoma cell line (Lemmon et al., 1982) was kindly provided by Dr. David Gottlieb, Washington University, St. Louis, MO. 1A6 is identical to monoclonal antibody 105 which has been shown to bind an extracellular epitope of NCAM but not to inhibit NCAM function as assayed by brain vesicle aggregation (Watanabe et al., 1986). Hybridoma cells secreting the monoclonal antibody A2B5 which recognizes gangliosides on the surfaces of neurons and type II astrocytes (Eisenbarth et al., 1979; Raff et al., 1983) were purchased from the American Type Culture Collection (Rockville, MD). For the

preparation of ascites fluid, hybridoma cells were injected intraperitoneally into 10-wk-old Balb/c female mice that had been primed 10 days previously with tetramethylpentadecane (pristane). CSAT IgG was purified from ascites fluid by Protein A Sepharose CL-4B chromatography as described in Neff et al. (1982), and 1A6 IgG was purified by ammonium sulfate precipitation followed by ion exchange chromatography on DEAE cellulose as described in Hudson and Hay (1980). Fab fragments of CSAT and 1A6 IgG were prepared by papain digestion followed by ion exchange chromatography on DEAE cellulose as described in Hudson and Hay (1980).

A rabbit antiserum to chick brain NCAM was generated as described in Bixby and Reichardt (1987); the antiserum recognizes all three forms of NCAM (ld, sd, and ssd) in immunoblots of brain membranes and inhibits adhesion and neurite outgrowth by chick peripheral neurons on skeletal myotubes in vitro (Bixby and Reichardt, 1987; Bixby et al., 1987). The anti-N-cadherin serum was generated in New Zealand White rabbits against a purified 90 kD proteolytic fragment of a 130 kD Ca^{++}-dependent CAM expressed by avian neural retinal cells (Crittenden et al., 1988). The anti-N-cadherin serum recognizes a single 130 kD protein in immunoblots of chick retinal membrane proteins separated by 2-dimensional gel electrophoresis (Crittenden et al., 1988). This polypeptide has been referred to in the literature as NcalCAM (Bixby et al., 1987; Crittenden et al., 1988) but is now known to crossreact with monoclonal antibodies to N-cadherin (S. Crittenden and J. Lilien, unpublished observations). Anti-NCAM and anti-N-cadherin IgG were prepared by ammonium sulfate precipitation followed by chromatography on DEAE (Hudson and Hay, 1980). Fab' fragments of these antibodies were prepared by pepsin digestion followed by reduction and alkylation as described in Hudson and Hay (1980).

Cell Culture: Since the integrin β_1 monoclonal antibody is highly species-specific, rat astrocytes were chosen as a substrate for neurite outgrowth by chick retinal neurons to

minimize the effects of this antibody on the astrocytes themselves. Similarly, the N-cadherin and NCAM antisera were generated against avian antigens, but do crossreact to some extent with rat antigens (K. Tomaselli, unpublished observations). Astrocytes were isolated from neonatal rat cortices as described (Fallon, 1985a) and >90% expressed the astrocyte marker, glial fibrillary acidic protein (GFAP). Of these GFAP-positive cells, 90-95% were flat, polygonal cells that resembled Type I astrocytes isolated from rat optic nerve; the remaining 5-10% were process-bearing and, like Type II astrocytes, expressed the cell surface gangliosides recognized by the A2B5 monoclonal antibody (Raff et al., 1983). Enzymatically dissociated embryonic day 7 (E7) and E11 chick retinal neurons were separated from non-neuronal cells by differential adhesion to tissue culture plastic and grown in defined medium as described in Hall et al. (1987). When the neurons were co-cultured with astrocytes, the defined medium was supplemented with 0.5% fetal calf serum as in Cohen et al. (1986).

Substrate Preparation and Coculture: In some experiments, astrocytes were cultured as small "island" monolayers (5mm in diameter) centered on 13 mm glass coverslips that had been previously coated with laminin (10 μg/ml in Ca^{2+}, Mg^{2+}-free PBS) as described in Tomaselli et al. (1986). For other experiments, coverslips were coated with 1 mg/ml poly-D-lysine (PDL) in water, washed with Ca^{2+}, Mg^{2+}-free PBS, and seeded with ~10^5 astrocytes, such that after 24-48 hours of culture, a confluent monolayer of astrocytes extended to the coverslip edge. In coculture experiments, ~10^5 retinal neurons were plated per coverslip and cultured for 16-20 h at 37°C in the absence or presence of antibodies. Antibodies were diluted in medium and sterile-filtered through 0.45 μm nitrocellulose filters (Millipore; Bedford, MA) before use.

Attachment Assays: Astrocytes (~20,000 cells/0.28 cm^2 well) were plated into 96-well tissue culture plates (Flow Laboratories; McLean, VA) that had been previously coated

with PDL. After 1-2 days of culture, retinal cell attachment to confluent monolayers of astrocytes was tested as follows: Retinal neurons were labeled for 3-5 h in methionine-free growth medium containing ^{35}S-methionine (100 μCi/ml). The labeled cells were pelleted by centrifugation at 1,000 xg for 5 min and resuspended in normal growth medium. ~50,000 cells (~500,000 cpm) were added to each well, and the plates were spun for 3 minutes at ~600 xg to ensure an even distribution of retinal neurons on the astrocyte monolayer. Cells were allowed to attach for 1 h at 37°C. Non-adherent retinal neurons were removed by adding 0.1 ml of medium to each side of the well and aspirating the supernatants. This wash procedure was sufficient to completely remove neurons from wells coated with the non-adhesive protein, bovine serum albumin. A more stringent wash was achieved by performing this wash procedure three times instead of once. Wells were then examined through a microscope to confirm that the astrocytes themselves were not being lost from the substrate. Retinal cells that adhered to the astrocyte monolayer were then extracted in 0.1 ml 1% SDS in PBS overnight, mixed with 3 ml Aquasol, and counted in a scintillation counter. In each experiment, all determinations were made in triplicate.

Analysis of Retinal Neurite Outgrowth on Laminin and Astrocyte Substrates: Retinal neuron-astrocyte cocultures were fixed in 3.7% formaldehyde and stained with the A2B5 monoclonal antibody as described in Tomaselli et al. (1986). Briefly, fixed cultures were blocked and permeabilized in 1% normal goat serum/0.05% saponin/PBS, and then incubated for 1 hour in a 1:500 dilution of A2B5 ascites fluid. After washing 5 times in PBS, cultures were incubated for 1 hour in a 1:200 dilution of rhodamine isothiocyanate-coupled goat anti-mouse second antibody (Cappel Laboratories, Malvern, PA). Coverslips were mounted with gelvatol and viewed with a 63x planopochromat oil immersion lens on a Zeiss inverted IM microscope equipped with rhodamine optics. Since the A2B5 monoclonal antibody vividly stains both the cell

bodies and processes of all avian neural retinal cells (Eisenbarth et al., 1979), the number of neurons bearing processes and the length of these processes could be determined. Type II astrocytes were also labeled by A2B5, but were easily distinguished from neurons on the basis of their fibrous morphology (see Raff et al., 1983). The percentage of A2B5 positive neurons with a neurite greater than two cell body diameters in length was counted. Between 100 and 200 neurons per coverslip were tabulated in this manner. To determine neurite lengths, processes that were visible from cell soma to growth cone were drawn by camera lucida. Drawings were traced onto a computerized digitizing pad (GTCO, Rockville, MD) which calculated their lengths. Retinal neurons usually extended a single, unbranched neurite. When a neuron had more than one process, the lengths of individual neurites were summed to give the total neuritic output per neuron. All values presented in the tables and figures, therefore, refer to the total neuritic output per neuron.

RESULTS

E7 Retinal Neurite Outgrowth on Laminin Substrates and Astrocyte Monolayers.

Neurite outgrowth by embryonic chick retinal neurons grown in vitro was examined by quantifying the percent of neurons bearing neurites and the total length of neuronal processes extended by each neuron. Retinal neurons from embryonic day 7 (E7) embryos were grown on substrates consisting of small "islands" of rat cortical astrocyte monolayers centered on glass coverslips that were coated with a concentration of laminin (10 μg/ml) that is optimal for attachment and neurite outgrowth by E7 retinal neurons (Hall et al., 1987). After 16-20 hours of culture, retinal neurons extended a profuse array of neurites on both the astrocyte monolayers and the surrounding laminin substrate (Fig. 3.1a and b), and neurites frequently crossed the border between the two substrates (Fig. 3.1c). Of the neuronal cell bodies in contact with the astrocyte monolayer, 41% extended neurites, compared to 32% of those in contact with the laminin substrate (Table 3.1). Comparison of the average lengths of retinal neurites extended on these substrates showed that those on astrocyte monolayers were ~2.2-fold longer than those on laminin (Table 3.1). Moreover, neurites at the astrocyte-laminin border appeared to prefer the astrocyte substrate, often following the edge of the astrocyte island before growing onto the laminin surround (Fig. 3.1c); neurites were rarely seen skirting the astrocytes in favor of the laminin substrate. These observations suggest that astrocyte cell surfaces are a more favorable substrate for process extension than laminin alone.

Neurite Outgrowth by E7 Retinal Neurons on Astrocyte Monolayers: Inhibition by N-cadherin and Integrin β_1 Antibodies.

To define the molecular interactions underlying E7 retinal neurite outgrowth on astrocyte surfaces, antibodies that recognize and inhibit the activity of avian neuronal

cell surface proteins were tested for their effects on astrocyte-stimulated neurite outgrowth. The monoclonal antibody 1A6, which recognizes an extracellular epitope of avian NCAM but does not inhibit any detectable NCAM function (Watanabe et al., 1986; Bixby and Reichardt, 1987; Bixby et al., 1987), was used to control for possible non-specific effects of antibody binding to cell surfaces. Compared to cultures grown in the absence of antibodies, neither 1A6 IgG nor 1A6 Fab significantly affected the percent of process-bearing neurons or the average length of neurites extended on either laminin or astrocytes (Table 3.2).

A monoclonal antibody (CSAT) that recognizes the avian β_1 subunit of the integrin family of ECM receptor heterodimers (Horwitz et al., 1985; Buck et al., 1986; reviewed in Hynes, 1987 and Ruoslahti and Pierschbacher, 1987), has previously been shown to eliminate retinal neuron attachment and process outgrowth on laminin, fibronectin, and collagen types I and IV (Hall et al., 1987; K. Neugebauer, unpublished observations). When E7 retinal neurons were grown on astrocyte monolayers in the presence of high concentrations of either anti-integrin β_1 IgG (0.1 mg/ml) or Fab fragments (0.25 mg/ml), the percent of neurons bearing processes was reduced by 25-50% as compared to 1A6 Fab-containing control cultures (Table 3.2). The average lengths of the remaining processes were significantly reduced by 15-30% (Table 3.2, Fig. 3.3). These effects on astrocyte-stimulated neurite outgrowth were relatively weak compared to the virtual elimination of neurite outgrowth by integrin β_1 antibodies on laminin (see Table 3.2). Since doubling the concentration of anti-integrin β_1 Fab to 0.5 mg/ml had only a slightly stronger effect on neurite outgrowth on astrocytes, the 0.25 mg/ml concentration of anti-integrin β_1 Fab used in these and subsequent experiments appears to have been saturating. As illustrated in Fig. 3.2, the overall morphology of neurons cultured in the presence of integrin β_1 antibodies resembled that of neurons in 1A6-containing cultures despite the inhibitory effects of integrin β_1 antibodies on neurite outgrowth.

Antibodies that recognize and inhibit the function of N-cadherin, a Ca^{++}-dependent cell adhesion molecule expressed on avian retinal neurons (Crittenden et al., 1988), had dramatic effects on several aspects of retinal neurite outgrowth on astrocyte monolayers. In the presence of 1 mg/ml anti-N-cadherin Fab's, the percent of process-bearing E7 retinal neurons was reduced by ~50% (Table 3.2). Neurites that did grow in the presence of anti-N-cadherin Fab's were visibly shorter than those seen in control cultures, reflecting a ~40% reduction in average neurite length (Table 3.2, Fig. 3.2c, Fig. 3.3). Often these neurites had enlarged growth cones (Fig. 3.2c). Doubling the concentration of anti-N-cadherin Fab' did not significantly enhance these effects, indicating that the effects of 1 mg/ml anti-N-cadherin Fab' were saturating (Table. 3.2). Interestingly, bivalent anti-N-cadherin IgG had stronger inhibitory effects than did monovalent Fab' fragments (Table 3.2). Inhibition of retinal neurite outgrowth by N-cadherin antibodies was substrate-specific, since neurite outgrowth on laminin substrates was not significantly affected by anti-N-cadherin IgG (Table 3.2).

To determine whether the inhibitory effects of integrin β_1 and N-cadherin antibodies were additive, E7 retinal neurons were cultured on astrocytes in the presence of both antibodies. A representative experiment shown in Table 3.3 demonstrates that the combined presence of integrin β_1 and N-cadherin bivalent antibodies resulted in a small but statistically insignificant increase in the inhibition observed with anti-N-cadherin alone.

When E7 retinal neurons were cultured on astrocytes in the presence of anti-NCAM Fab' fragments at concentrations as high as 2 mg/ml, no parameter of neurite outgrowth examined was significantly affected. Instead, neurons treated with anti-NCAM Fab' were indistinguishable from control on the basis of morphology (Fig. 3.2d), neurite length distribution (Fig. 3.3), percent of neurons with neurites or average neurite length (Table 3.2). Even when added with anti-N-cadherin Fab', anti-NCAM Fab's did not produce a decrease in neurite outgrowth beyond the effects of anti-N-

cadherin alone (Table 3.3). Thus, we were unable to detect any effects of anti-NCAM Fab' on E7 retinal neurite outgrowth on astrocytes. However, in cultures treated with 1 mg/ml anti-NCAM IgG, a ~50% decrease in the average neurite length was observed (Table 3.2). As our preparation of anti-NCAM Fab's is known to block the function of chick NCAM (Bixby and Reichardt, 1987; Bixby et al., 1987), the effects of bivalent NCAM antibodies in the present experiments seem most likely to be due to crosslinking of their target antigen rather than a specific inhibition of NCAM function. Interestingly, NCAM IgG binding only inhibited process outgrowth on astrocytes, as it had no significant effects on process outgrowth on laminin substrates (Table 3.2).

Attachment of E7 Retinal Neurons to Astrocyte Monolayers

To determine whether the inhibition of neurite outgrowth by either integrin β_1 or N-cadherin antibodies was correlated with a reduction in retinal neuron attachment to astrocytes, the attachment of ^{35}S-methionine-labeled E7 retinal neurons to confluent monolayers of astrocytes was measured in the absence or presence of antibodies. In each experiment, neurons contacting the astrocyte monolayers were subjected to two wash conditions differing in stringency (see Materials and Methods). The results in Fig. 3.4 show that after one wash of the monolayer (open bars), neither anti-integrin β_1 IgG, anti-NCAM Fab', nor anti-N-cadherin IgG significantly reduced retinal neuron attachment to astrocytes when applied individually. The combination of all three antibodies decreased attachment by ~25% compared to control under these conditions (Fig. 3.4).

After a more extensive washing procedure (3 washes), only anti-N-cadherin IgG had effects when applied alone, reducing attachment by 30% (Fig. 3.4; stippled bars). While integrin β_1 antibodies did not enhance the inhibitory effects of N-cadherin antibodies, the combination of integrin β_1, N-cadherin, and NCAM antibodies did reduce attachment by an additional 30% (Fig. 3.4). Thus, NCAM appears to potentiate

the inhibition of attachment by N-cadherin antibodies. This result, in combination with the observed insensitivity of retinal neurite outgrowth to NCAM antibodies, suggests that NCAM function may be more important for adhesion than for neurite outgrowth by E7 retinal neurons.

Process Outgrowth by E11 Retinal Neurons on Astrocytes: Inhibition by N-cadherin, Integrin β_1 and NCAM Antibodies

The possibility that retinal neurons of different developmental ages use different mechanisms for neurite outgrowth on astrocytes was examined using E11 retinal neurons in the coculture paradigm used for E7 neurons. Unlike E7 retinal neurons, E11 retinal neurons do not attach or extend processes on either laminin or fibronectin, although they continue to do so on collagens and astrocytes (Cohen et al., 1986; Hall et al., 1987). E11 retinal neurons grown on astrocytes for 16-20 hours exhibited diverse morphologies, ranging from cells with large somata and many bifurcating processes to cells with small somata and one or two straight neurites (Figs. 3.5a and b). Spatulate growth cones with multiple filopodia were not characteristic of E11 retinal neurons as they were for E7 retinal neurons (fig. 3.5). Thus, older retinal neurons grown on astrocytes regenerate growth cones that resemble those observed in the optic nerve and tract in situ (Bovolenta and Mason, 1987). Although fewer E11 than E7 neurons extended processes (29% versus 41%; Tables 1 and 4), E11 neurites were comparable to E7 neurites in average length (~200 μm; see Figs. 3.3 and 3.6). E11 neurite lengths were more heterogeneous in that approximately twice as many E11 as E7 neurons had either very short (<100 μm) or very long (>500 μm) neuritic arbors (compare Figs. 3.3 and 3.6). However, we were unable to correlate neurite length with neurons grouped by morphological characteristics. Similarly, the effects of antibodies on E11 neurite outgrowth (see below) could not be attributed to differential effects on neurons with distinct morphologies (data not shown).

Anti-N-cadherin Fab' fragments inhibited E11 neurite outgrowth on astrocytes to a similar extent as on E7 retinal neurons. Compared to 1A6 Fab, anti-N-cadherin Fab's consistently reduced the percent of process-bearing E11 neurons by ~50%, and the average length of remaining neurites by ~35% (Fig. 3.6, Table 3.4). Figure 3.6 shows that anti-N-cadherin increases the proportion of neurites shorter than 100 mm from 35% to 65%. In the presence of anti-N-cadherin, E11 retinal neurons appeared well attached to the astrocytes, although their neurites were visibly shorter (Fig. 3.5c).

When anti-integrin β_1 Fab fragments were tested, both the average neurite length and the percent of process-bearing neurons were reduced by ~30% (Table 3.4, Fig. 3.6). About 50% of the neurites in integrin b1-containing cultures were shorter than 100 μm (Fig. 3.6). These effects of anti-integrin β_1 Fabs were not anticipated, as E11 retinal neurons have lost their integrin-dependent responsiveness to purified laminin and fibronectin (Hall et al., 1987).

In contrast to that of E7 retinal neurons, E11 retinal neurite outgrowth on astrocytes was sensitive to anti-NCAM Fab' fragments in the medium. E11 retinal neurons extended neurites in the presence of 1 mg/ml anti-NCAM Fab's (Fig. 3.5d), but the percent of process-bearing neurons was reduced by ~50% and the average length of neurites was reduced by ~25% (Table 3.4). Figure 6 shows that anti-NCAM Fab' fragments virtually eliminated long neurites in the cultures, as ~90% of the neurites were shorter than 175 mm. Thus, these experiments show that inhibitory effects of anti-NCAM on retinal neurite outgrowth on astrocytes depends on the developmental age of the retinal neurons.

DISCUSSION

Our observations of chick retinal neurite outgrowth on astrocytes in the presence of antibodies to neuronal cell surface proteins support the following major conclusions: (1) The ability of embryonic chick retinal neurons to extend neurites rapidly when cultured on astrocyte cell surfaces depends strongly on the function of the Ca^{2+}-dependent cell adhesion molecule, N-cadherin, and to a lesser extent, on members of the integrin family of ECM protein receptor heterodimers; (2) NCAM's role in mediating retinal neurite outgrowth on astrocytes depends on the developmental age of the retinal neurons, such that E11 but not E7 neurons require NCAM function for maximal neurite extension. (3) Retinal neurons use additional receptors for attachment and neurite outgrowth on astrocytes, since neither was completely inhibited by any individual antibody or combination of antibodies tested. (4) The molecules used by retinal neurons for process outgrowth on astrocytes are similar to those used by peripheral ciliary ganglion neurons but differ in two important respects: retinal neurite outgrowth relies on (i) at least one additional CAM (NCAM) and (ii) integrin β_1-class ECM receptors even at developmental ages at which they have lost their responsiveness to purified laminin.

N-cadherin is a 130 kD neuronal cell surface protein that is important in the calcium-dependent aggregation of avian retinal neurons (Grunwald et al., 1982). N-cadherin mediates peripheral neurite outgrowth on cultured myotubes, Schwann cells and astrocytes (Bixby et al., 1987; 1988; Tomaselli et al., 1988b). N-cadherin is likely to act by a homophilic binding mechanism (i.e. neuronal N-cadherin binding to astrocyte N-cadherin), since a closely related epithelial cell CAM, E-cadherin, has been shown to function, at least in part, in this way (Nagafuchi et al., 1987). It is unclear whether any of the effects of N-cadherin antibodies observed in these experiments can be attributed to the binding of antibodies to astrocyte N-cadherin; however, since we have shown that

saturating levels of anti-N-cadherin were used (see Table 3.2), we are confident that N-cadherin on the neuronal surface was maximally inactivated. Here we have shown that N-cadherin antibodies strongly reduced both E7 and E11 retinal neurite outgrowth on astrocytes. The extent of this inhibition was similar for neurons of both ages; in the presence of anti-N-cadherin Fab' fragments, ~50% fewer E7 and E11 neurons grew neurites, and neurites in both cultures were 35-40% shorter than those that grew in control cultures. The growth cones of E7 retinal neurons often appeared enlarged in the presence of N-cadherin but not other antibodies. E11 retinal neurites did not bear spatulate growth cones under any of the conditions tested. N-cadherin is also involved in E7 retinal neuron adhesion to astrocytes, as determined by quantitative attachment assays. However, even under the most stringent conditions of our assay, N-cadherin antibodies reduced attachment by only ~30%, an amount insufficient to account for the potency of their effects on process outgrowth. The importance of N-cadherin in retinal neuron process outgrowth on astrocytes and its presence in the developing chick neural retina and optic nerve (Hatta et al., 1986, 1987), suggest that N-cadherin also promotes axonal extension on glial surfaces within the primary visual pathway.

Members of the integrin family of ECM receptors also mediate process outgrowth by neurons on astrocytes. The integrin β_1 subunit (Hynes, 1987; Ruoslahti and Pierschbacher, 1987) is shared by cell surface glycoprotein heterodimers that bind laminin and fibronectin directly (Horwitz et al., 1985; Akiyama et al., 1986; Buck et al., 1986; K. Tomaselli, unpublished observations). Anti-integrin β_1 monoclonal antibodies inhibit attachment and neurite outgrowth by a variety of avian neurons on substrates coated with purified laminin, fibronectin, and collagen types I and IV (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986; Cohen et al., 1987; Hall et al., 1987; K. Neugebauer, unpublished observations). Since integrin β_1 antibodies also inhibit neurite outgrowth on substrates coated with a laminin-heparan sulfate proteoglycan complex secreted by astrocytes as well as on intact ECMs (Tomaselli et al., 1986; 1988b; Cohen

et al., 1987), they were expected to prevent retinal neuron interactions with the fibronectin and laminin that rat cortical astrocytes are known to express in vitro (Liesi et al., 1983; 1986; Price and Hynes, 1985).

Anti-integrin β_1 Fab fragments reduced the percent of process-bearing E7 retinal neurons and the average neurite length by ~30% each. These effects are comparable to those of integrin β_1 antibodies on ciliary ganglion neurite outgrowth on astrocytes (Tomaselli et al., 1988b). Integrin β_1 antibodies did not significantly inhibit adhesion of retinal neurons to astrocytes in our assays, even though these antibodies virtually eliminate the attachment of retinal neurons to laminin, fibronectin, and collagen I and IV substrates (Hall et al., 1987; K. Neugebauer, unpublished observations). Thus, integrin receptor interactions with ECM components expressed on astrocyte surfaces do not appear to play a major role in neuron-astrocyte attachment.

Anti-integrin β_1 Fab fragments inhibited E7 and E11 retinal neurite outgrowth on astrocytes to the same extent. E11 retinal neurons have previously been shown to lose their ability to attach to and extend neurites on laminin and fibronectin substrates (Cohen et al., 1986; 1987; Hall et al., 1987). However, they retain integrins on their surface and their ability to attach to collagen types I and IV in an integrin-dependent manner (Cohen et al., 1987; Hall et al., 1987; K. Neugebauer, unpublished observations). Thus, the present results suggest that astrocytes express ECM proteins in addition to laminin and fibronectin (eg. collagen types I and IV) whose neuronal receptor(s) includes the integrin β_1 subunit. Alternatively, E11 retinal neurons may recover their response to laminin and/or fibronectin when grown in contact with astrocyte cell surfaces. In this respect, the behavior of retinal neurons differs from that of ciliary ganglion neurons: neurite outgrowth by these peripheral neurons on astrocytes is not inhibited by integrin β_1 antibodies at a developmental age (E14) when they, like E11 retinal neurons, no longer attach or extend neurites on purified laminin (Tomaselli et al., 1988b).

Anti-NCAM Fab' fragments did not affect any parameter of E7 neurite outgrowth on astrocytes even when added in combination with N-cadherin antibodies (cf. Table 3.3). Anti-NCAM Fab' alone did not detectably inhibit E7 retinal neuron attachment to astrocyte monolayers. Anti-NCAM Fab' did, however, have significant effects on attachment when added in combination with N-cadherin and integrin β_1 antibodies. Together, these results suggest that NCAM plays a relatively minor role in E7 retinal neuron interactions with astrocytes. In contrast, the same anti-NCAM Fab's dramatically inhibited neurite outgrowth by developmentally older (E11) retinal neurons: the percent of process-bearing E11 neurons was decreased by 50% and the average length of E11 neurites was decreased by 25%. Since homophilic NCAM binding in liposome assays is highly dependent on its concentration in the membrane (Hoffman and Edelman, 1983), the striking developmental change in the sensitivity of retinal neurite outgrowth to NCAM antibodies may reflect the two-fold increase in NCAM levels observed in the retina between E5 and E10 (Daniloff et al., 1986). Alternatively, changes in the molecular form of NCAM on the surface of the neurites may explain these findings: although sialic acid-poor M_r 180k and M_r 140k forms of NCAM predominate in both E5 and E10 retina, there is an apparent transition from sialic acid-rich to poor forms in the optic nerve between these ages (Schlosshauer et al., 1984).

The inhibition of E7 retinal neurite outgrowth on astrocytes by anti-NCAM IgG is the only instance in our experiments in which an IgG had effects not seen with Fab' fragments. Specifically, both IgG and Fab's generated from the same NCAM antiserum used here reduce ciliary ganglion neurite elongation on skeletal myotubes (Bixby et al., 1987), while neither is inhibitory on astrocytes or Schwann cells (Tomaselli et al., 1988b; Bixby et al., 1988). One possible explanation for these results is that in the process of crosslinking NCAM on the surface of retinal neurons, NCAM IgG redistributes other relevant proteins (eg. N-cadherin) in the plane of the membrane. The

cell adhesion molecule L1/NgCAM has been shown to co-redistribute with the 180 kD form of NCAM on neural membranes after treatment with bivalent anti-NCAM IgG (Thor et al., 1986), establishing a precedent for this possibility.

Since neither attachment nor neurite outgrowth by retinal neurons on astrocytes was completely inhibited by the antibodies used in this study (see Fig. 3.4 and Table 3.3), adhesive molecules in addition to N-cadherin, integrin β_1 heterodimers, and NCAM must be active. It is possible that this activity represents a novel adhesive mechanism. However, three previously identified molecules that mediate neuronal interactions with astrocytes are also good candidates: (1) cytotactin, an ECM glycoprotein complex present in the optic fiber and inner plexiform layers of the embryonic chick retina (Crossin et al., 1986); (2) AMOG (adhesion molecule on glia), an integral membrane protein that promotes cerebellar granule cell adhesion to astrocytes (Antonicek et al., 1987); and (3) L1/NgCAM (Grumet and Edelman, 1988), a cell adhesion molecule present in the developing optic pathway (Daniloff et al., 1986; Lemmon and McLoon, 1986). L1 has been implicated in neurite outgrowth on other neurites and on Schwann cells (Chang et al., 1987; Bixby et al., 1988), and purified L1 promotes neurite extension when used as a culture substrate (Lagenaur and Lemmon, 1987). It is unlikely that all neurons use such additional mechanisms, since ciliary ganglion neurite outgrowth on astrocytes can be nearly completely accounted for by the combined activity of N-cadherin and the integrin β_1 heterodimers (Tomaselli et al., 1988b).

The above results suggest roles for integrins, N-cadherin, and NCAM in process outgrowth and adhesion in the developing retinotectal pathway. The early chick neural retina consists of a sheet of neuroepithelial cells that gradually differentiate into the various retinal cell types, beginning with the appearance of the first post-mitotic retinal ganglion cells at embryonic day 2.5-3 (Kahn, 1974). These cells migrate to the most vitreal portion of the retina and extend axons toward the optic stalk, using a laminin-

rich basal lamina and Muller glial endfeet as growth substrates (Rager, 1980; Easter et al., 1984; Cohen et al., 1987). At E7, ganglion cell axon initiation peaks (Halfter et al., 1983), and other post-mitotic retinal cell types are migrating to appropriate layers of the neural retina (Kahn, 1974). Although the ordered growth of axons to the optic stalk relies on the integrity of the basal lamina (Halfter et al., 1984; 1986), directional cues for growth are not provided by the intact, isolated basal laminae (Halfter et al., 1987). Our observations of E7 retinal neurite outgrowth on astrocyte surfaces implicate N-cadherin as well as integrin-class ECM receptors in axonal extension at this early developmental stage, raising the possibility that N-cadherin on glial endfeet may guide advancing axons to the optic stalk.

Axonal growth within the optic nerve peaks between E7 and E11 and occurs primarily along the endfeet of astroglial precursors and preexisting axonal surfaces (Rager, 1980; Silver and Rutishauser, 1984). Laminin may be involved in the elongation of the first ganglion cell axons to enter the optic pathway (E3), but it disappears from the optic nerve by E7 (Cohen et al., 1987). NCAM is localized at the surface of glial endfeet in the nerve where it may play a role in axon extension (Silver and Rutishauser, 1984; Thanos et al., 1984). The observed developmental change in NCAM's role in retinal neurite outgrowth on astrocytes is consistent with the hypothesis that axonal growth in the optic pathway becomes progressively more dependent on cell surfaces.

At the optic tectum, retinal ganglion cell axons synapse with a topological specificity that generates a tectal map of the visual field. Neuronal recognition via differential adhesion (chemoaffinity) has long been thought to account for this targeting phenomenon (Sperry, 1963). Since glial endfeet line the developing optic nerve fiber layer of the tectum (Lemmon, 1985; 1986; Bork et al., 1987), they may provide cues for axon growth and guidance. The sensitivity of astrocyte-stimulated E11 retinal neurite outgrowth to NCAM antibodies is consistent with evidence that NCAM is involved in accurately targeting axons at the tectum (Fraser et al., 1984). As N-cadherin and

integrins remain active on the surface of E11 retinal neurons, it will be of interest to determine whether these receptor systems also regulate synaptogenic events at the optic tectum.

TABLE 3.1: Neurite outgrowth by E7 retinal neurons on laminin and astrocytes.

substrate	% with neurites	avg. length (μm)
laminin (10 μg/ml)	32 +/- 5 (6)	110 +/- 13 (33)
astrocytes	41 +/- 6 (4)	243 +/- 18 (76)

E7 retinal neurons were grown on astrocyte "islands" centered on laminin-coated coverslips for 16-20 hours. Neurons were stained with A2B5 antibody followed by a rhodamine conjugated second antibody, and the percentage of labeled neurons with a neurite greater than two cell diameters was determined for the number of coverslips indicated in parentheses. Fluorescent neurites were drawn by camera lucida, and the total neurite length per neuron was determined using a computerized digitizing pad. The number of neurites measured is indicated in parentheses. All values represent the mean +/- SEM. The difference between percent of cells with neurites is not significant ($p > .05$, student's t test); the difference between average neurite length is significant ($p < .0005$).

Table II. Effects of Antibodies on E7 Neurite Outgrowth

Antibody	Astrocytes		Laminin	
	Percent with neurites	Percent average length*	Percent with neurites	Percent average length*
None	41 ± 6 (4)	119 ± 11 (60)‡	32 ± 5 (6)	109 ± 11 (33)
1A6				
1 mg/ml IgG	42 ± 5 (4)	100 ± 3 (72)	27 ± 3 (5)	100 ± 10 (42)
1 mg/ml Fab	42 ± 4 (5)	100 ± 5 (128)		
Integrin β_1				
0.10 mg/ml IgG	20 ± 1 (3)	71 ± 6 (40)§	1 ± 1 (3)	–
0.25 mg/ml Fab	32 ± 6 (3)	81 ± 5 (91)§	8 ± 4 (3)	ND
0.50 mg/ml Fab	22 (1)	85 ± 9 (37)§		
N-cadherin				
1 mg/ml IgG	9 ± 2 (4)	40 ± 4 (51)§	24 ± 3 (8)	100 ± 10 (29)
1 mg/ml Fab'	22 ± 5 (4)	61 ± 4 (93)§		
2 mg/ml Fab'	19 (1)	52 ± 6 (42)§		
NCAM				
1 mg/ml IgG	25 ± 3 (4)	56 ± 5 (67)§	27 ± 5 (8)	103 ± 11 (25)
1 mg/ml Fab'	38 ± 5 (4)	93 ± 6 (95)‡		
2 mg/ml Fab'	32 (1)	109 ± 11 (33)‡		

E7 retinal neurons were cultured for 16–20 h on either confluent astrocyte monolayers or on laminin in the absence or presence of antibodies. After fixation and immunostaining with A2B5, the percent of neurons bearing neurites was determined as described in Materials and Methods; the number of cultures examined is indicated in parentheses. Fluorescently labeled neurites were traced and their lengths determined as described in Materials and Methods; the number of neurites measured is indicated in parentheses. All values represent the mean ± SEM; note that neurite lengths are expressed as percent of 1A6 control. The average neurite length on laminin in the presence of 1A6 IgG is 101 ± 10 μm, and on astrocytes in the presence of 1A6 Fab is 191 ± 9 μm. The Student's *t* test was used to assign significance levels to differences in average neurite length from 1A6 controls as indicated.

* Relative to 1A6 control.

‡ Not significantly different from 1A6 control ($P > 0.05$).

§ Significantly different from 1A6 control ($P < 0.005$).

Table 3.3

Table III. Effects of Combined Antibodies on E7 Retinal Neurite Outgrowth on Astrocytes

Antibody	Percent with neurites	Percent average length*
1A6 IgG	40 ± 8 (2)	100 ± 10 (39)
Integrin IgG	19 ± 1 (2)	63 ± 8 (40)‡
N-cadherin IgG	9 ± 3 (2)	37 ± 5 (50)‡
Integrin + N-cadherin	5 ± 2 (1)	33 ± 6 (14)‡§
1A6 Fab	48 (2)	100 ± 9 (30)
NCAM Fab'	44 ± 1 (2)	92 ± 13 (32)
N-cadherin Fab'	29 ± 1 (2)	61 ± 9 (30)‡
NCAM + N-cadherin	26 (1)	64 ± 9 (25)‡§

E7 retinal neurons were grown for 16 h on confluent astrocyte monolayers in the presence of the indicated antibodies. All antibodies were added to a final concentration of 1 mg/ml, except anti-integrin β_1 IgG was used at 0.1 mg/ml. After the cultures were fixed and stained, the percent of neurons bearing neurites was determined (n = the number of cultures examined) and the neurite length was measured as described (n = the number of neurites traced). All values represent the mean ± SEM. The Student's t test was used to assign significance levels to differences in average neurite lengths from 1A6 controls as indicated.

* Relative to 1A6 control.

‡ Significantly different from 1A6 control ($P < 0.0005$).

§ Not significantly different from anti-N-cadherin IgG alone ($P > 0.05$).

Table 3.4

Table IV. Antibody Effects on E11 Retinal Neurite Outgrowth on Astrocytes

Antibody	Percent with neurites	Percent average length*
None	29 ± 3 (4)	117 ± 10 (109)
1A6 Fab	32 ± 6 (4)	100 ± 8 (110)‡
Integrin Fab	20 ± 1 (2)	67 ± 8 (65)§
NCAM Fab'	16 ± 3 (4)	76 ± 7 (117)§
N-cadherin Fab'	15 ± 1 (2)	64 ± 8 (48)§

E11 retinal neurons were cultured on confluent astrocyte monolayers for 16–20 h in the absence or presence of Fab' fragments. All Fab fragments were added to a final concentration of 1 mg/ml, except anti-integrin β_1 was used at 0.25 mg/ml. A2B5-stained neurons were scored for the percent with neurites (n = the number of cultures examined). The lengths of fluorescent neurites were determined as described in Materials and Methods (n = the number of neurites measured). All values represent the mean ± SEM. The Student's t test was used to compare differences in average neurite length to 1A6 control.

* Relative to 1A6 control.

‡ Not significantly different from 1A6 control ($P > 0.05$).

§ Significantly different from 1A6 control ($P < 0.05$).

Figure 3.1: E7 retinal neurite outgrowth on astrocytes and laminin. Neurons were cultured for 16 h, fixed, and labeled with the A2B5 monoclonal antibody followed by a rhodamine-conjugated second antibody. (a) retinal neurites on a laminin substrate; (b) neurites on a confluent astrocyte monolayer; and (c) a higher magnification view of neurite outgrowth at the border of a single astrocyte (outlined by arrows) surrounded by laminin. Note that the neurites tend to follow the edge of the astrocyte before fasciculating and growing onto the laminin. Scale bars: 50 μm (a and b) and 10 μm (c).

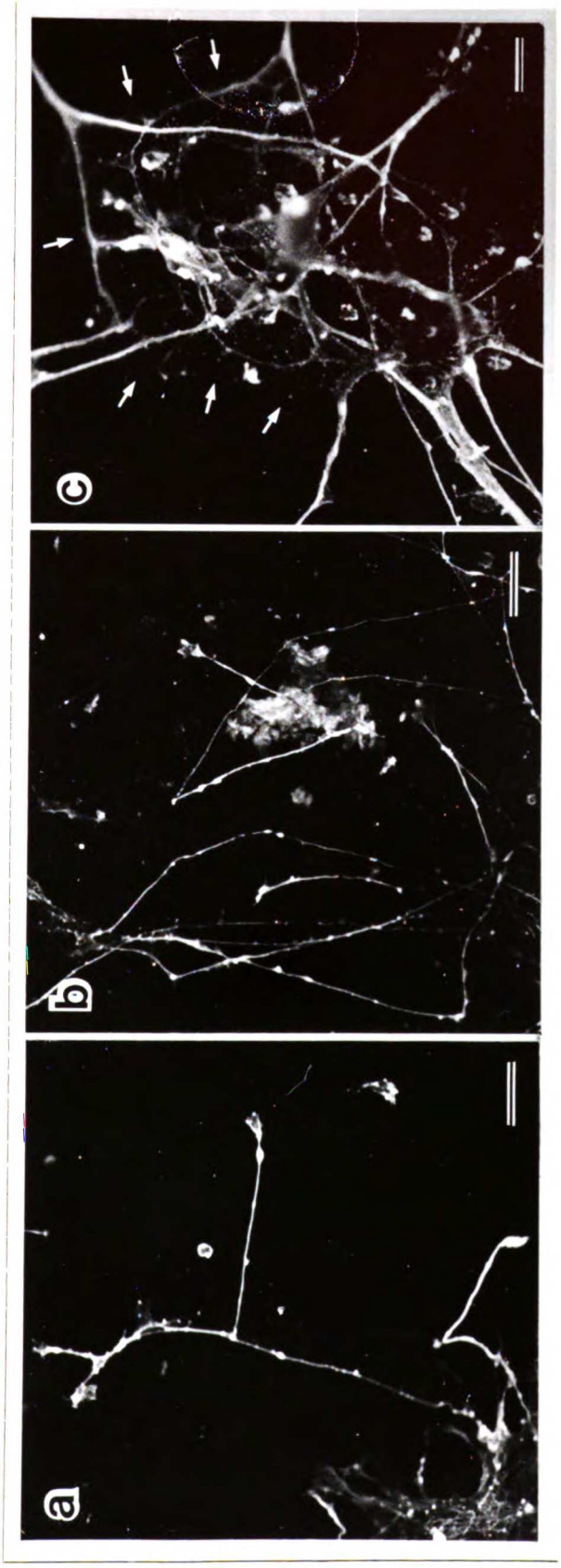

a
b
c

Figure 3.2: E7 retinal neurite outgrowth on astrocyte monolayers in the presence of antibodies to neuronal cell surface proteins. Neurons were cultured on astrocytes for 16 h in the presence of antibodies, fixed and stained with A2B5 antibody. (a) Neurite outgrowth in the presence of 1 mg/ml 1A6 Fab fragments is extensive: portions of three long neurites traverse the field. (b) In the presence of 0.25 mg/ml anti-integrin β_1 Fab, neurites do grow but are shorter on average. (c) This single neuron grown in the presence of 1 mg/ml anti-N-cadherin IgG is quite short (~120 μm) and has two growth cones (arrowheads). (d) Neurite outgrowth in the presence of 2 mg/ml anti-NCAM Fab' is not visibly affected; portions of four long neurites crisscross the field. Examples of retinal cell bodies (arrows) and growth cones (arrowheads) are visible in each field. Scale bar: 10 μm.

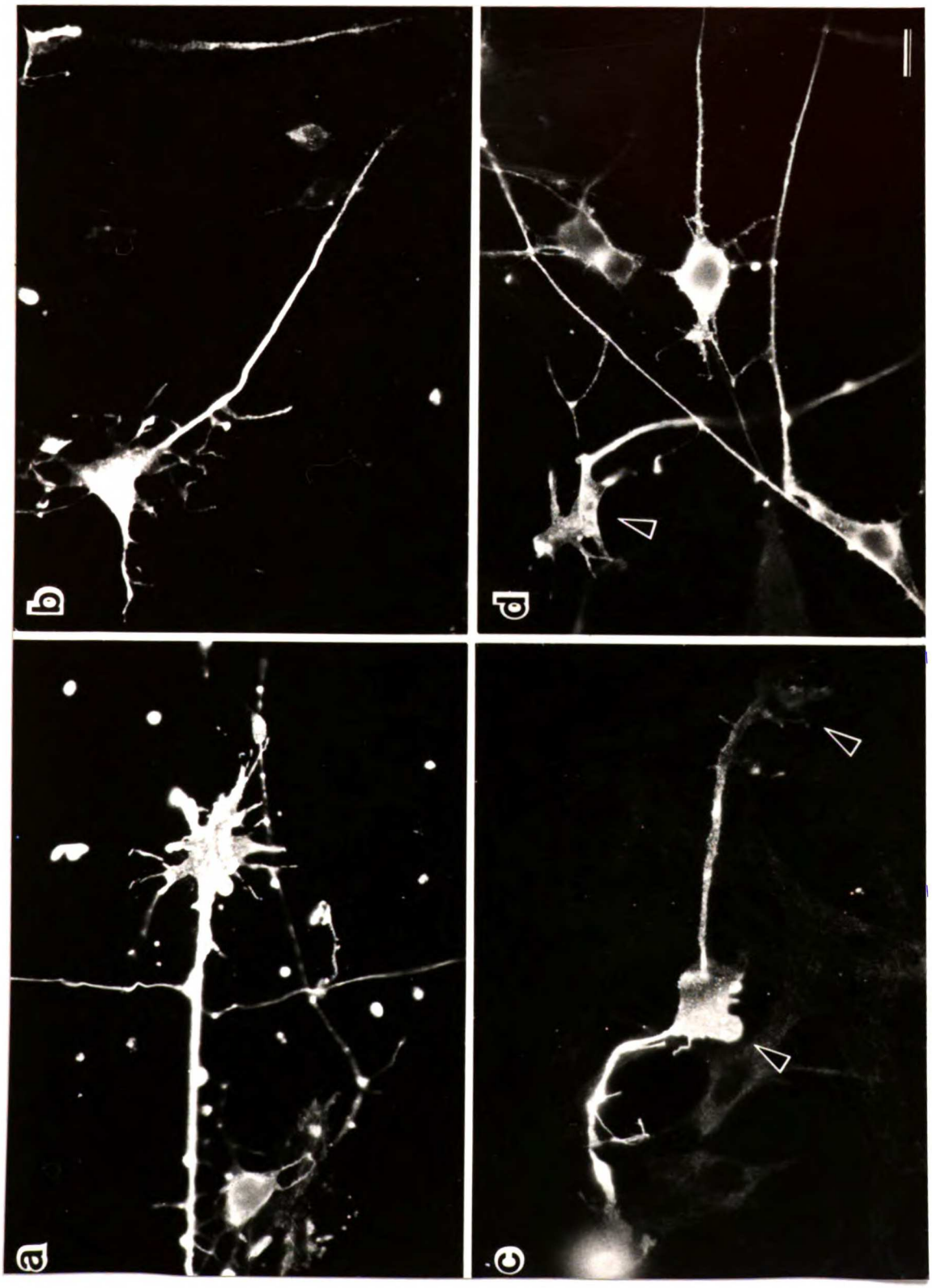

a
b
c
d

Figure 3.3: Effects of antibodies on E7 retinal neurite length on astrocytes after 16-20 h of culture. Distribution of neurite lengths in the presence of the following Fab fragments: closed circles, 1 mg/ml 1A6 Fab (n=128 neurites); open triangles, 0.1 mg/ml integrin β_1 Fab (n=91); open circles, 1 mg/ml NCAM Fab' (n=95); closed triangles, 1 mg/ml N-cadherin Fab' (n=93). Neurite lengths were binned by 25 μm intervals and plotted versus the percentage of neurites measured that were longer than the length (x) indicated. The distribution of neurite lengths in the presence of 1A6 Fab was identical to that in the absence of antibodies (omitted for clarity). Inset: histogram of average neurite lengths (mean +/- SEM) in the absence or presence of Fab fragments of antibodies. 60 neurites were measured for the "no antibody" condition. Data from four separate experiments were pooled to yield the final distribution curve and histogram.

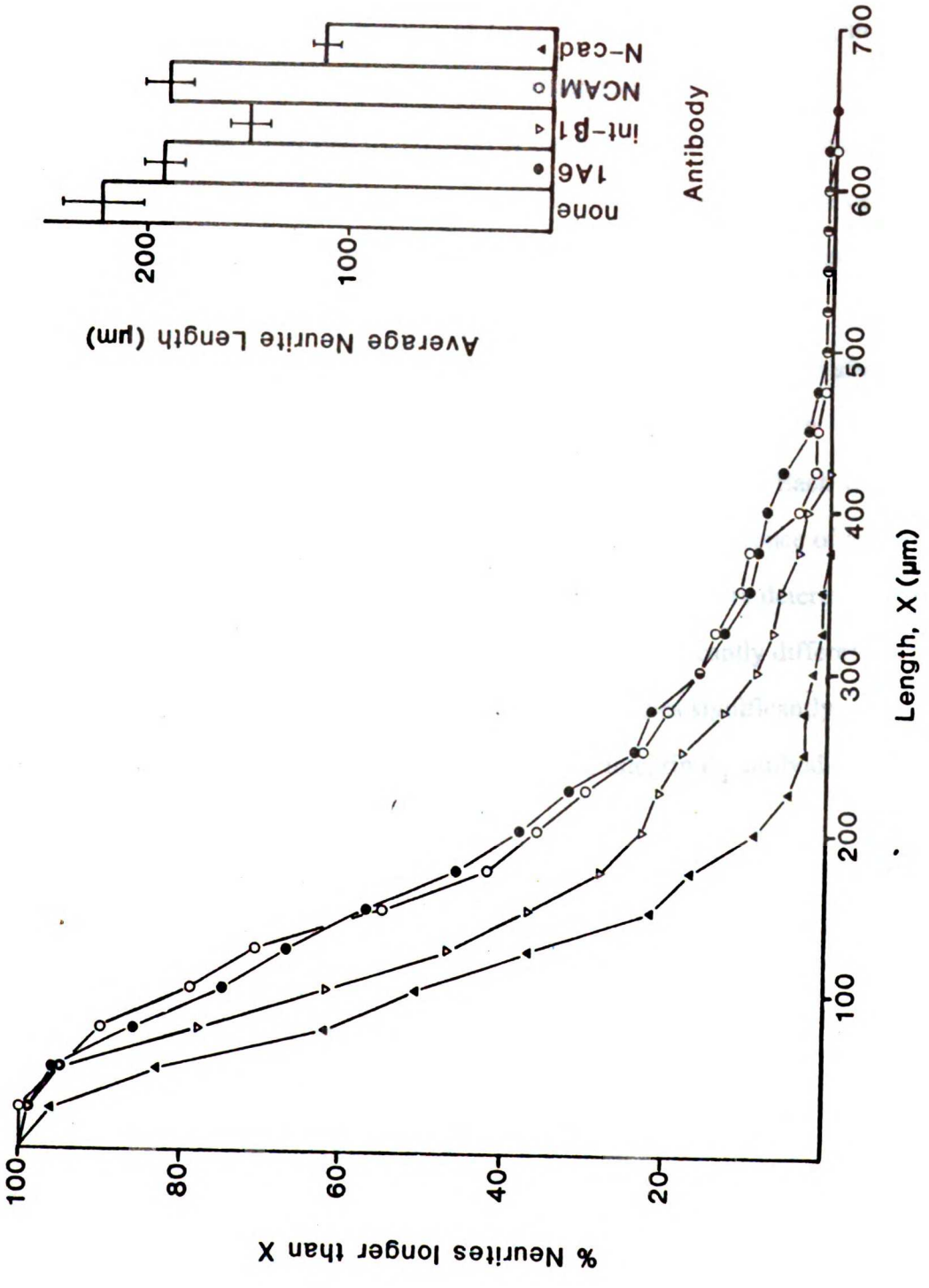

% Neurites longer than X
100
80
60
40
20
100
200
300
400
500
600
700
Length, X (μm)
Average Neurite Length (μm)
200
100
none
1A6
int-β1
NCAM
N-cad
Antibody

Figure 3.4: Attachment of ^{35}S-labeled E7 retinal neurons to astrocyte monolayers in the absence or presence of antibodies. All antibodies indicated are IgG, except anti-NCAM which is Fab'.

Antibodies were added to a final concentration of 1 mg/ml, except that anti-integrin β_1 was used at 0.1 mg/ml. Neurons were and allowed to attach to astrocyte monolayers for 1 h. Wells were subjected to either one (open bars) or three (stippled bars) washes. Radioactivity associated with adherent neurons was quantitated by extracting the cells with 1% SDS and counting the extracts in a scintillation counter. Under both assay conditions, 80-100% of the counts added to each well was associated with the monolayer in absence of antibody, although fewer counts were usually detected after the additional washing procedure. Triplicate determinations were made in each experiment, and the results of 3-6 experiments per variable were averaged. Each value is expressed as a percent of cpm remaining with the astrocytes in the presence of control 1A6 IgG (mean +/- SEM). Significant differences from 1A6 control were determined by the student's t test (*, $p < .005$); bars without asterisks are not significantly different ($p > .05$). Note that under extensive wash conditions, NCAM antibodies significantly decreased attachment beyond the effects of N-cadherin plus integrin β_1 antibodies ($p< .05$).

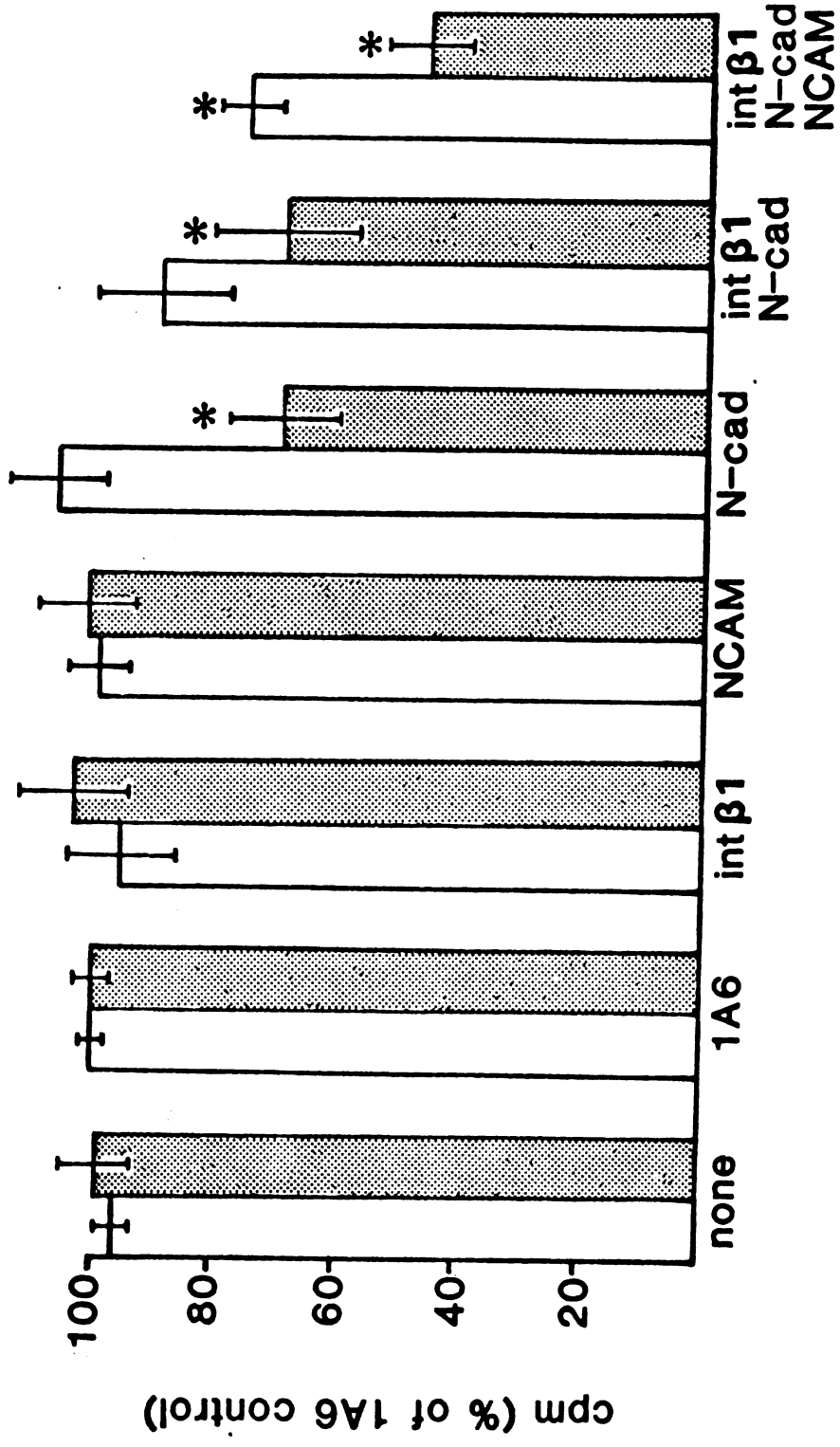

cpm (% of 1A6 control)
100
80
60
40
20
none
1A6
int β1
NCAM
N-cad
int β1
N-cad
int β1
N-cad
NCAM
ANTIBODY
*
*
*
*
*

Figure 3.5: E11 retinal neurite outgrowth on astrocytes after 16 h of culture in the absence and presence of antibodies. Neurons are fluorescently stained with A2B5 antibody. (a) and (b) Two distinct neuronal morphologies in the absence of antibodies. Note that the neurites in (b) extend out of the field. (c) In the presence of 1 mg/ml anti-N-cadherin Fab', many neurons lack neurites, and the neurites present are short and often wispy. (d) In the presence of 1 mg/ml anti-NCAM Fab', neurites are obviously shortened. Scale bar: 10 μm.

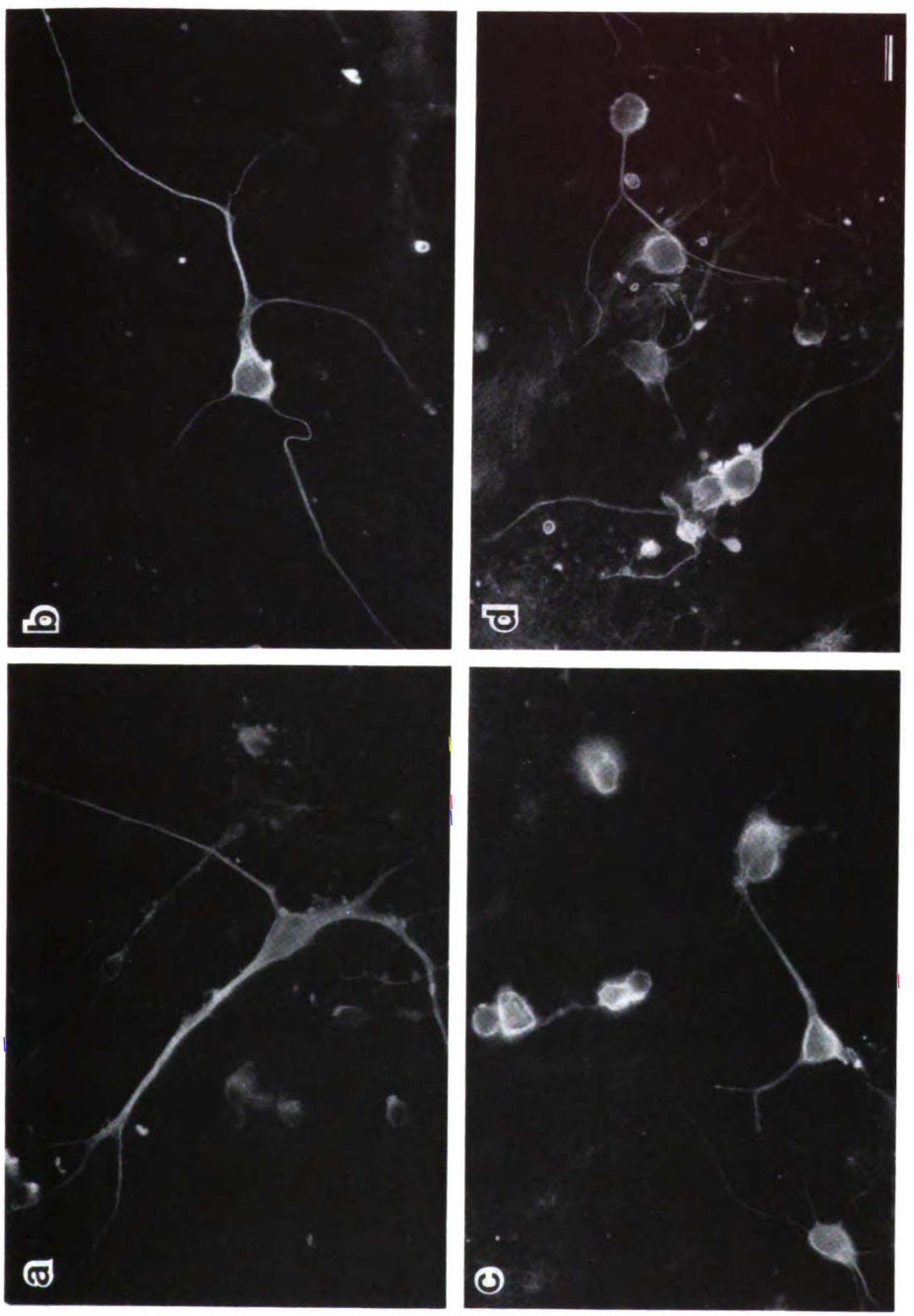
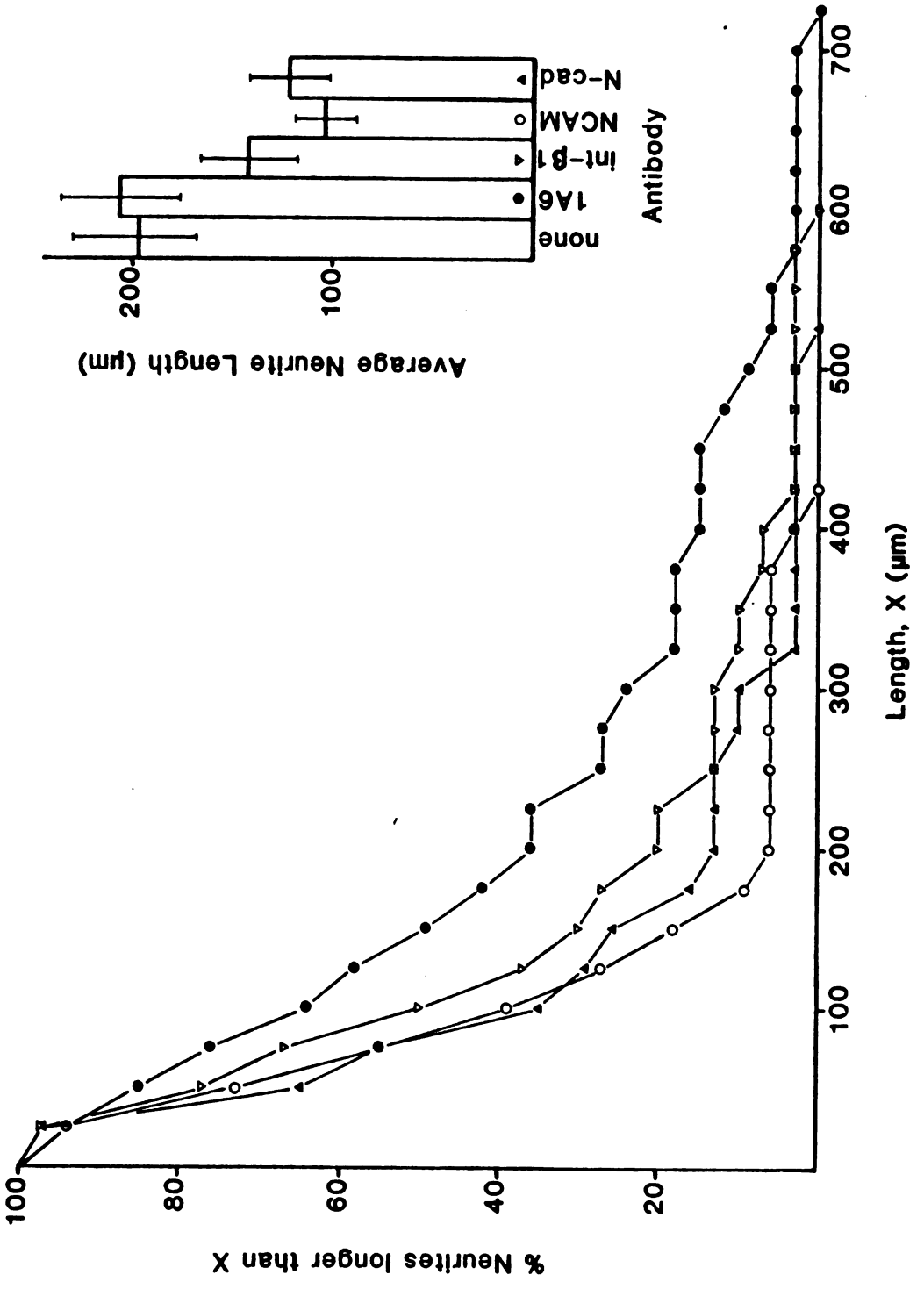

b
d
a
c

Figure 3.6: Effects of antibodies on E11 retinal neurite length on astrocytes after 16 h of culture. Distribution of E11 neurite lengths in the presence of the following Fab fragments: closed circles, 1 mg/ml 1A6 Fab (n=33); open triangles, 0.25 mg/ml anti-integrin β_1 Fab (n=30); open circles, 1 mg/ml anti-NCAM Fab' (n=33); closed triangles, 1 mg/ml anti-N-cadherin Fab' (n=31). Inset: histogram of average neurite lengths (mean +/- SEM) in the absence or presence of Fab fragments. 33 neurites were measured for the "no antibody" condition. Data from a representative experiment was used to generate the distribution curve and histogram.


% Neurites longer than X
100
80
60
40
20
100
200
300
400
500
600
700
Length, X (μm)
Average Neurite Length (μm)
200
100
none
1A6
int-β1
NCAM
N-cad
Antibody

CHAPTER FOUR

β_1 Integrin Activity Promoted by a Monoclonal Antibody: Implications for Regulated Function

Abbreviations used in this chapter: VN, vitronectin; FN, fibronectin; Col, collagen; LN, laminin; FBG, fibrinogen.

SUMMARY

Integrins are a family of heterodimeric receptors that mediate cell adhesive events in response to extracellular ligands. A monoclonal antibody (TASC) against the integrin β_1 subunit was isolated for its ability to inhibit cell interactions with vitronectin. Unexpectedly, TASC was found to promote cell adhesion to other β_1-class integrin ligands, providing evidence that two structural domains of β_1 are involved in ligand binding. Further, inactive receptors gain function in the presence of TASC, suggesting that the activity of β_1-class integrins is subject to modulation at the cell surface.

INTRODUCTION

On many cell types, the physiological effects of integrin binding to ligand include the regulation of cell adhesion, shape, motility, migration, intracellular signalling and gene expression (Akiyama et al., 1990; Hemler, 1990; Springer, 1990). Information regarding the structure, function and regulated expression of integrin heterodimers is, therefore, central to understanding the complex role of these receptors in a diverse set of cellular events. From a family of at least 12 α and 6 β subunits, individual α and β subunits associate non-covalently to form unique integrin heterodimers with distinct ligand-binding specificities and cellular consequences of receptor occupancy. The β_1 subunit is widely expressed on animal cells and can couple with at least 7 α subunits to produce heterodimers that bind laminin (LN), collagens, fibronectin (FN), VCAM, and vitronectin (VN) (Akiyama et al., 1990; Hemler, 1990; Bodary and McLean, 1990). On the surface of developing neurons, β_1-class integrins are likely to play a prominent role in axon extension and pathfinding (Reichardt and Tomaselli, 1991). Early embryonic chick retinal neurons express β_1-class integrins that mediate adhesion and neurite outgrowth in vitro on purified LN, Col IV, and FN (Hall et al., 1987). Neuronal responses to each of these proteins are inhibited by the CSAT monoclonal antibody which recognizes the integrin β_1 subunit (Buck et al., 1986). Here we show that retinal neurons also adhere to VN but in a CSAT-insensitive manner.

We have isolated a monoclonal antibody against the integrin β_1 subunit that inhibits cell adhesion to VN, indicating that a β_1-class integrin heterodimer(s) does act as a neuronal VN receptor. In contrast to the effects of CSAT, this antibody promotes adhesion to collagens, LN, FN, and FBG. We have named this antibody TASC to indicate that its activity is the inverse of CSAT's. In addition, we show that the epitopes recognized by TASC and CSAT are non-overlapping. Thus, the complementary effects of TASC and CSAT implicate the involvement of two

distinct epitopes on the β_1 subunit in forming active ligand-binding domains. Further, cells that do not respond to a particular purified ligand (LN) in vitro are shown to do so in the presence of TASC. These experiments suggest that β_1-class integrins expressed on the cell surface may occupy multiple affinity states that can be modulated.

RESULTS

TASC inhibits adhesion to vitronectin

Previous studies have shown that retinal neurons bind astrocyte monolayers even in the presence of antibodies that inhibit the function of β_1-class integrins (CSAT) and other non-integrin cell-cell adhesion molecules (Neugebauer et al., 1988), implicating the activity of additional receptors in neuronal responses to cell surface proteins. Thus, we assayed for retinal neuron adhesion to another purified integrin ligand, VN, that is recognized by several classes of integrin receptors (Akiyama et al., 1990). After 1 hour, 100% of embryonic day 7 (E7) chick retinal neurons attached to VN (Fig. 4.1a). Attachment to VN was completely blocked by the Arg-Gly-Asp-containing hexapeptide GRGDSP but not by the monoclonal antibody CSAT (Fig. 4.1a) at concentrations previously shown to completely inhibit the attachment of these cells to LN, FN, and Col IV (Hall et al., 1987).

A monoclonal antibody screen was initiated in an effort to identify the neuronal receptor(s) for VN, using the attachment assay to select clones of interest. IgG secreted by the TASC clone inhibited retinal neuron attachment to VN by ~25% in a 1 hour assay, and the addition of CSAT did not enhance this effect (Fig. 4.1a). Since the strength of cell attachment to ligand-coated substrates increases with time (Lotz et al., 1989), a shorter term assay was carried out to determine whether greater effects of TASC could be detected. In 10 minutes, ~40% of the cells attached to VN in the absence of antibodies; TASC decreased attachment by ~35%, and weak effects of CSAT were observed both alone and in combination with TASC (Fig. 4.1a). Together, TASC and CSAT did not completely block attachment of these cells to VN at either time point, suggesting that additional receptors for VN are expressed by these cells.

To explore the effects of TASC on another cell type, VN-mediated adhesion

was studied using the chicken myeloblast cell line HD11 (Beug et al., 1979). Within 5 minutes, 70% of HD11 cells attached to VN in an RGD-dependent manner (Fig. 4.1b). HD11 attachment to VN was decreased ~65% by TASC and ~50% by CSAT. The doses of each antibody were saturating, since neither effect was enhanced by increasing antibody concentrations in the medium (data not shown). The effects of TASC and CSAT were additive, blocking nearly all HD11 binding to VN.

TASC recognizes the integrin β_1 subunit

To further characterize the TASC antigen as a likely VN receptor, ^{35}S-labeled extracts of E7 retinal neurons were immunoprecipitated with TASC IgG. The set of proteins precipitated by TASC was essentially identical to material precipitated by CSAT (Fig. 4.2a). Both antibodies precipitated proteins co-migrating with the mature (110kD) and immature (100kD) forms of the β_1 subunit (Fig. 4.2a, lane 7; Akiyama et al., 1990). Proteins of Mr 145 and 135k were co-precipitated and represent associated a subunits previously characterized on retinal neurons (Hall et al., 1987). Similarly, TASC and CSAT precipitated identical patterns of proteins from extracts of HD11 cells (data not shown).

To determine whether TASC and CSAT recognize the same set of β_1-class heterodimers, extracts of E7 retinal neurons (Fig 4.2a) were depleted of antigen by sequential immunoprecipitation with either antibody. Subsequent precipitation of these depleted extracts revealed that all of the TASC antigen had been removed by CSAT and vice versa. The antigen is unlikely to have been degraded during the course of the experiment, since another cell surface glycoprotein, the integrin α_v subunit, was readily detected by immunoprecipitation from an aliquot of the same depleted sample (Fig. 4.2a, lane 6). TASC also bound to its antigen on an immunoblot of retinal and HD11 cell extracts (Fig. 4.2b), further indicating that TASC directly binds the β_1 subunit.

Since TASC and CSAT had different effects on cell binding to VN, it seemed likely that the two antibodies recognize distinct epitopes on the β_1 subunit. To test this possibility, retinal neuron attachment to antibody-coated substrates was measured in the absence and presence of competing antibodies in the medium. The results in Table 4.1 show that while CSAT competed for attachment to CSAT and TASC competed for attachment to TASC, neither antibody inhibited attachment to the other. The monoclonal antibody, JG22, served as an important control, since it has already been shown to bind an epitope which overlaps with CSAT's (Horwitz et al., 1984). Indeed, JG22 competed for binding to CSAT and vice versa; JG22 did not compete for binding to TASC (Table 4.1). Thus, CSAT and TASC recognize non-overlapping epitopes on the integrin β_1 subunit.

TASC promotes attachment to other β_1-class integrin ligands

To examine the effects of TASC on cell-binding to other β_1-class integrin ligands, we assayed retinal neuron and HD11 cell attachment in several ways. First, E7 retinal neuron attachment to collagen I was time-dependent and blocked by CSAT (Fig. 4.3a). TASC IgG accelerated E7 retinal neuron attachment to Col I: whereas about 70% of these cells adhered to Col I in one hour in the absence of TASC IgG, the same level of adhesion was achieved in the presence of TASC in 5 minutes (Fig. 4.3a). This effect was b_1-class integrin-specific, since CSAT in combination with TASC abolished all binding. Fab fragments of TASC were just as effective in promoting early adhesion to Col I (Fig. 4.3a), indicating that the TASC-mediated increase in binding was not due to bivalent antibody-induced crosslinking of receptors.

Secondly, E7 retinal neuron attachment to substrates coated with varying concentrations of collagen I was measured in the absence and presence of TASC. TASC lowered the dose-dependence of attachment by about 4-fold (Fig. 4.3b).

Further, TASC did not promote attachment to very low concentrations of ligand or to the BSA-blocked substrate, excluding the possibility that TASC itself had been adsorbed to the plastic surface.

Cell interactions with other β_1-class integrin ligands were also enhanced by TASC. E7 retinal neuron attachment to LN was increased ~5-fold and to collagen IV ~3-fold by both bivalent and monovalent TASC in short term assays (Fig. 4.3c). Since nearly 100% of these cells attach to each ligand after 1 hour (Hall et al., 1987), TASC-mediated increases in cell binding represent an acceleration of adhesion as seen on Col I. On both substrates, TASC-enhanced attachment was blocked by CSAT. Although β_1-class integrin receptors for fibrinogen (FBG) are expressed by some cells (see below), E7 retinal neurons did not attach to FBG in the absence or presence of TASC (Fig 4.3c). Thus, TASC does not promote attachment by transforming the specificity of β_1-class integrin heterodimers expressed by a particular cell. Rather, TASC increases the efficacy of ligand-specific binding by integrins that are already expressed.

HD11 cell attachment to the 120kD cell-binding fragment of fibronectin (FN120) and Col I was also promoted by TASC, and elevated binding to both substrates was blocked by CSAT (Fig. 4.3d). In contrast to the example of retinal neurons, TASC also promoted the attachment of HD11's to FBG (Fig. 4.3d). This was surprising, since previously characterized cell binding to FBG has been attributed to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins rather than β_1-containing heterodimers (Pytela et al., 1986; Cheresh, 1987; Languino et al., 1989). CSAT blocked the TASC-induced HD11 attachment to FBG (Fig. 4.3d), and in experiments where HD11 attachment to FBG was detectable in the absence of TASC, CSAT alone was effective in blocking FBG binding (data not shown). Thus, the β_1 subunit in association with an unknown α subunit expressed by HD11 cells comprises a previously undescribed receptor for FBG.

TASC unmasks the activity of apparently non-functional receptor(s)

Purified laminin is a potent promoter of neurite outgrowth by early embryonic chicken retinal neurons in vitro, and both neuronal attachment and neurite outgrowth are abolished by the CSAT antibody (Hall et al., 1987). With increasing developmental age, these responses to laminin are lost, such that E11 retinal neurons do not attach or extend neurites on purified LN (Hall et al., 1987). However, CSAT inhibits neurite extension by older as well as younger neurons on astrocyte monolayers where the cell adhesion molecules NCAM and N-cadherin are also active (Neugebauer et al., 1988). This suggests that although integrins are present on E11 neurons and are functional in situations where multiple neurite-promoting receptors are engaged, they are not able to transduce neurite-promoting signals in response to purified ligands in vitro. To test the possibility that E11 retinal neurons continue to express integrin receptors for LN, these neurons were assayed for their ability to attach to purified LN in the presence of TASC IgG. Figure 4.4 shows that E11 retinal neurons attached to LN within 10 minutes in the presence of TASC, and that this induced attachment was blocked by CSAT. This observation argues that the developmental regulation of integrin function on these cells is not achieved by the simple downregulation of receptor expression. Instead, the observed developmental changes must be due, at least in part, to mechanisms which lead to the reversible inactivation of receptor populations on the cell surface.

DISCUSSION

TASC, a monoclonal antibody against a novel site on the integrin β_1 subunit, has been identified and shown to have the remarkable ability to inhibit cell adhesion to one ligand, vitronectin, while promoting cell attachment to other β_1-class integrin ligands. The identification of TASC has facilitated experiments that demonstrate (i) the function of β_1-class integrin heterodimers in cell attachment to VN and FBG, (ii) the existence of two distinct sites on the β_1 subunit involved in forming ligand-binding domains, and (iii) the likelihood that β_1-class integrin heterodimers exist on the cell surface in multiple affinity states that can be modulated.

TASC inhibited the attachment of E7 retinal neurons and HD11 myeloblasts to VN, establishing a role for β_1-class integrins as VN receptors. One such heterodimer, $\alpha_v\beta_1$, has been shown to act as a VN receptor on some cells (Bodary and McLean, 1990). However, neuronal attachment to VN was not blocked by a monoclonal antibody against the β_1-subunit (CSAT) that inhibits cell interactons with collagens, LN, and FN. In fact, CSAT had weak effects on retinal neuron attachment to VN that were only detectable very early in the process of adhesion. On other cell types, such as HD11's and chicken embryo fibroblasts (Buck and Horwitz, 1987), CSAT does have significant inhibitory activity on VN. The variable CSAT-sensitivity of adhesion to VN may reflect cell type-specific expression of multiple VN receptors, perhaps in different combinations. In addition to $\alpha_v\beta_1$, three VN-binding integrin heterodimers have been identified, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, and platelet $\alpha_{IIb}\beta_3$ (Akiyama et al., 1990; Hemler, 1990; Pytela et al., 1986; Cheresh et al., 1989). $\alpha_v\beta_3$ is synthesized by both retinal neurons and HD11's (chapter 5; appendix B), but it is not known whether either cell expresses β_5. It is also possible that multiple β_1-class VN receptors with different antibody sensitivities exist.

In contrast to its effects on VN, TASC promoted the attachment of retinal neurons and HD11's to other β_1-class integrin ligands, LN, FN, and collagens. This

unusual property enabled us to detect the presence of β_1-class integrin receptors for FBG on HD11 cells. Previously characterized integrin receptors for FBG do not include the β_1 subunit, but rather β_3 (Pytela et al., 1986; Cheresh, 1987; Languino et al., 1989). The identity of the α subunit which associates with β_1 to produce FBG binding is unknown and currently under investigation.

The nature of TASC-induced cell attachment suggests that TASC directly increases the affinity of β_1-class integrins for LN, FN, collagens, and FBG. TASC caused neurons to attach to col I more quickly and with a lower concentration-dependence of ligand. TASC also promoted the rapid attachment of neurons to LN and col IV as well as HD11s to FN120 and col I. TASC Fab fragments were equally effective in promoting cell attachment, excluding the possibility that TASC functions by crosslinking integrins. It is unlikely that TASC induces the expression of new receptors on the cell surface, either by the insertion of receptors from internal pools or by the induction of new protein synthesis, since many of the effects were detectable at very short time points. For example, HD11 attachment to FN120 was increased two-fold at "0-time" which allows only 2 minutes for the cells to interact with antibody. Finally, TASC did not alter the ligand-binding specificity of β_1-class integrin heterodimers: retinal neurons did not attach to FBG even at long time points or in the presence of TASC. Since a β_1-class integrin heterodimer has been implicated in HD11 binding to FBG, retinal neurons must not express this heterodimer nor any other that can be activated by TASC to bind FBG. Thus, TASC promotes ligand-specific cell attachment, implicating the activation of individual $\alpha\beta$ integrin dimers as the basis for TASC's effects.

The experiments presented here document the distinctive effects of CSAT and TASC, two monoclonal antibodies that bind non-overlapping epitopes on the integrin β_1 subunit. In Figure 4.5, we propose a model of integrin structure and function which predicts two distinct structural domains of the β_1 subunit involved

in ligand-binding. In the absence of antibodies, cell surface integrins bind "ligand 1" (LN, FN, FBG, and collagens) as well as "ligand 2" (VN) depending on the expression of specific α subunits, represented here as a composite α subunit. CSAT interferes with a site on β_1 required for the binding of ligand 1 but not for ligand 2. TASC binds a distinct site on β_1 that is required for the binding of ligand 2 only. Occupancy of this site by TASC results in an activation of the site perturbed by CSAT, such that ligand 1 is bound with a higher affinity. CSAT continues to recognize and perturb β_1 function, and thus even activated receptors are unable to bind ligand. Note that an alternate model constructed around one ligand binding site would require multiple conformations of that domain in order to explain how TASC simultaneously inhibits ligand 2 binding and promotes ligand 1 binding. Distinct epitopes on the integrin α_4, α_{IIb}, and β_3 subunits have been implicated in the binding of multiple ligands (Santoro and Lawing, 1987; Elices et al., 1990; Frelinger et al., 1990), lending further support to the notion that more than one ligand binding site may exist on individual integrin heterodimers.

The primary structures of β subunits 1-6 have been determined by molecular cloning and reveal homology between the six predicted protein products, including a pattern of four cysteine-rich repeats in the extracellular domains (Ramaswamy and Hemler, 1990; Suzuki and Naitoh, 1990; Hogervorst et al., 1990; Sheppard et al., 1990). Little is known about the domains required for α subunit association or ligand binding. Thus far, ligand binding by β_1-class integrins can be divided into two sets, one sensitive to short amino acid peptides containing the arg-gly-asp sequence (VN, FN, and FBG (appendix B)) and a second unaffected by RGD (LN and collagens) (Akiyama et al., 1990). In the cases of $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, radiolabeled RGD is crosslinked primarily to the β_3 subunit rather than to the α subunit, and peptide mapping places the RGD binding domain amino-terminal of the cysteine-rich repeats (Smith and Cheresh, 1988; D'Souza et al., 1988). Within

this region, a stretch of 21 amino acids is highly conserved among the sequenced b subunits (Ramaswamy and Hemler, 1990; Suzuki and Naitoh, 1990; Hogervorst et al., 1990; Sheppard et al., 1990). However, the epitopes bound by CSAT and TASC cannot be mapped onto β_1 using this information, since there is not a strict correlation between antibody and RGD inhibition. Thus, the placement of CSAT and TASC binding sites on β_1 in Fig 4.5 is arbitrary. Since TASC recognizes denatured antigen on an immunoblot, mapping of its epitope may be possible by generating TASC-reactive proteolytic peptide fragments.

Our model implies that the affinity of β_1-class integrin-ligand binding can be modulated at the cell surface, suggesting that this type of regulation has physiological relevance. This seems to be the case for developing chick retinal neurons. At early times in development (E5-E7), 100% of retinal neurons adhere to LN and more than half extend long neurites rapidly on LN in culture; both responses are abolished by the CSAT antibody (Hall et al., 1987). With increasing developmental age (E8-E12), retinal neurons lose the ability to attach or extend neurites on purified LN (Hall et al., 1987). Two observations suggest that LN receptors do not disappear from the cell surface: (i) immunoprecipitation of β_1-class integrins from cells of both ages does not reveal the simple loss of a subunit(s) (Hall et al., 1987) and (ii) CSAT inhibits neurite extension by older as well as younger neurons on astrocyte monolayers where multiple receptor systems, including the cell adhesion molecules NCAM and N-cadherin, are also active (Neugebauer et al., 1988). TASC's ability to induce E11 retinal neuron attachment to purified LN demonstrates the continued cell surface expression of LN-binding integrins at levels sufficient to support cell attachment. Consistent with this, E11 retinal ganglion cells (a subpopulation of the cells used here) express fewer LN binding sites than do E6-7 retinal ganglion cells, and the remaining LN binding sites are of lower affinity (Cohen et al., 1989).

Perhaps the activation state of cell surface β_1-class integrins reflects conformational differences in β_1 subunit structure determined by the clustering of heterogeneous cell surface receptors, receptor association with the cytoskeleton, and/or covalent modification. Precedence for each of these mechanisms has been established for integrins expressed by blood cells which require strict regulation of cell-cell and cell-matrix interactions in order to function with a high degree of specificity. For example, the avidities of platelet $\alpha_{IIb}\beta_3$ and lymphocyte LFA-1 (a β_2-class integrin) dramatically increase upon cell activation (Dustin and Springer, 1989; Plow and Ginsberg, 1989). Conformational changes in each of these receptors have been invoked to explain such modulation, and these are likely to depend on intracellular signalling mechanisms (Springer, 1990; Dustin and Springer, 1989; Plow and Ginsberg, 1989; Figdor et al., 1990). Recent reports show that β_1-class integrin binding on immune cells is also upregulated by activation without a change in the levels of receptor expression (Shaw et al., 1990; Shimizu et al., 1990). On activated macrophage, for example, increased $\alpha_6\beta_1$ binding to LN is coincident with the phosphorylation of the a_6 subunit and a greater association of the dimer with the cytoskeleton (Shaw et al., 1990). The results presented here suggest that β_1-class integrin receptor avidity can also be modulated on non-circulating cells whose movements within tissues may require rapid and dynamic regulation. The TASC antibody will be a valuable reagent in dissecting the molecular mechanisms which control β_1-class integrin affinity and function.

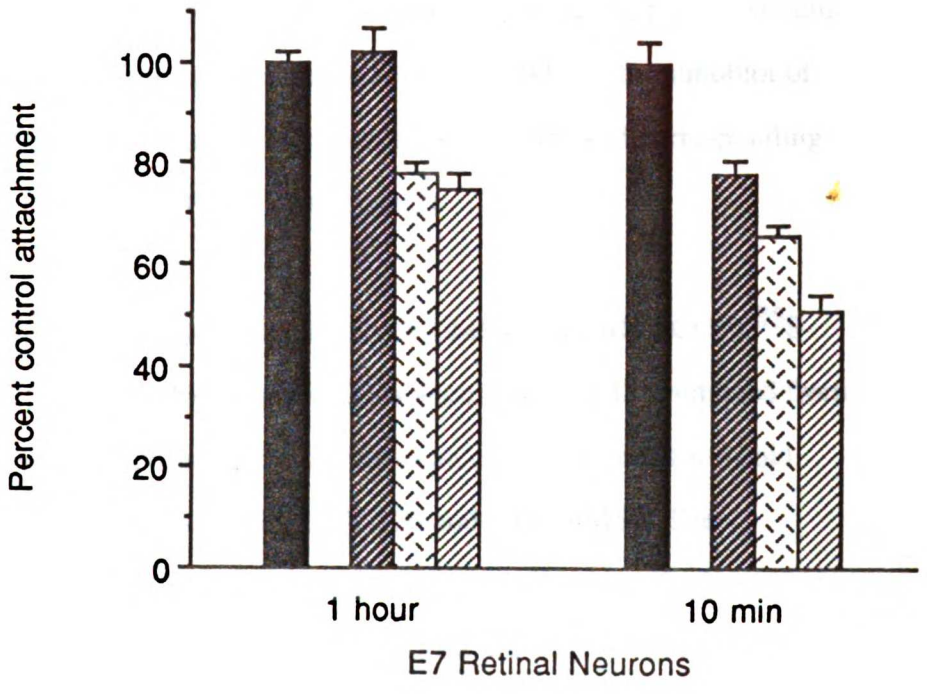
Table 4.1: E7 retinal neuron attachment to substrates coated with 5 mg/ml CSAT, TASC, and JG22 antibodies (see Fig. 4.1 for methods) was measured. Cells were incubated with the competing antibody for 2 min at room temperature, spun onto the antibody substrates, incubated for 5 min and washed. The results (mean +/- SEM) are normalized to the attachment observed to each antibody in the absence of competing antibody. The SEM for control attachment to CSAT was 14%, to JG22 8%, and to TASC 11%.

Table1. Antibody competition of adhesion to antibody substrates

Substrate antibody	Competing antibody % control attachment		
	CSAT	JG22	TASC
CSAT	7+/-2	7+/-2	120+/-18
JG22	5+/-2	10+/-2	131+/-13
TASC	96+/-11	90+/-11	5+/-1

Figure 4.1: TASC inhibits cell attachment to vitronectin. *a.* E7 retinal neuron attachment to VN for 1 hour is inhibited by 100 mg/ml GRGDSP and 200mg/ml TASC but not by 100 mg/ml CSAT. CSAT has no effect even in combination with TASC. After only 10 minutes, E7 retinal neuron attachment to VN is inhibited by 200 mg/ml TASC and 50 mg/ml CSAT. CSAT potentiates the effect of TASC slightly. b. HD11 myeloblast attachment to VN is inhibited by GRGDSP, TASC, and CSAT. The effects of TASC and CSAT are additive. In all cases, attachment is normalized to that seen in the absence of antibody. 100% of E7 retinal neurons adhere to VN in 1h and ~40% in 10 min. ~70% of HD11 cells adhere to VN in 5 min.

METHODS. VN was purified from fetal bovine serum by chromatography on Heparin Sepharose Cl-6b (Pharmacia) (Yatogo et al., 1988). IgG's were purified from ascites fluid on Protein A-Sepharose Cl-4b (Pharmacia), and GRGDSP was from Collaborative Research. Cell attachment was measured as per Hall et al. (1987) with modifications: 96-well plates were coated with 75 µl/well of 2 µg/ml bovine VN in PBS overnight at 4°C. Wells were blocked with 1% BSA in PBS for 3 h at 37°C to eliminate non-specific binding. Dissociated embryonic day 7 chick retinal neurons were prepared as described (Hall et al., 1987). HD11 cells were harvested with 0.05% trypsin/0.02% EDTA, spun down, and resuspended in RPMI. 100,000 cells of either type were seeded in each well, and the plates were spun at 48g for 2 min. After incubation at 37°C for the indicted times, non-adherent cells were washed from the substrate. Adherent cells were stained with crystal violet and quantitated by A_{540} (Bodary et al., 1989). In each experiment, all determinations were done in triplicate. To isolate TASC, integrin-rich chick brain glial cells were used as the immunogen for monoclonal antibody production in mice (see chapter 5). Clonal supernatants were screened for effects on retinal neuron attachment to VN.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40
20
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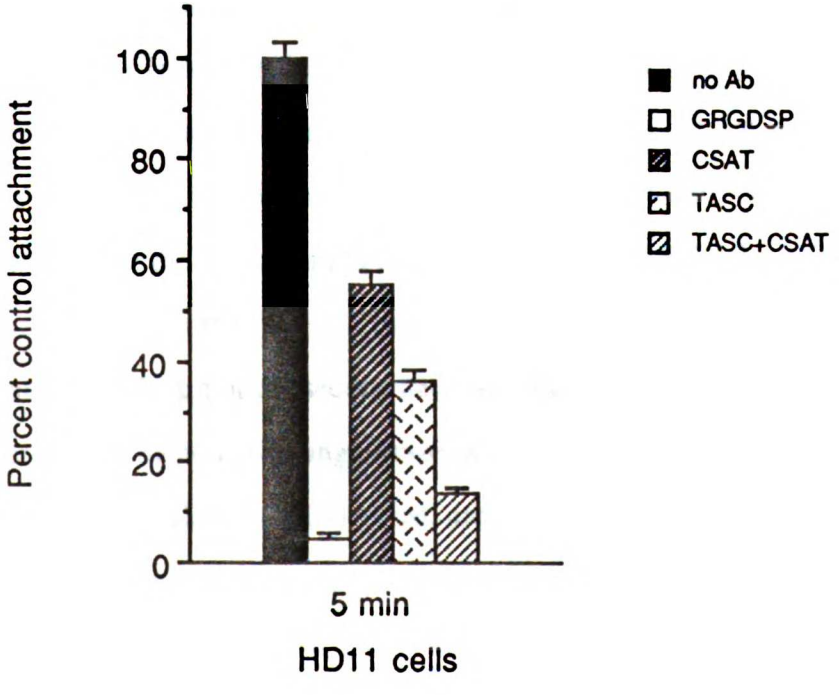
1 hour
10 min
E7 Retinal Neurons


Percent control attachment
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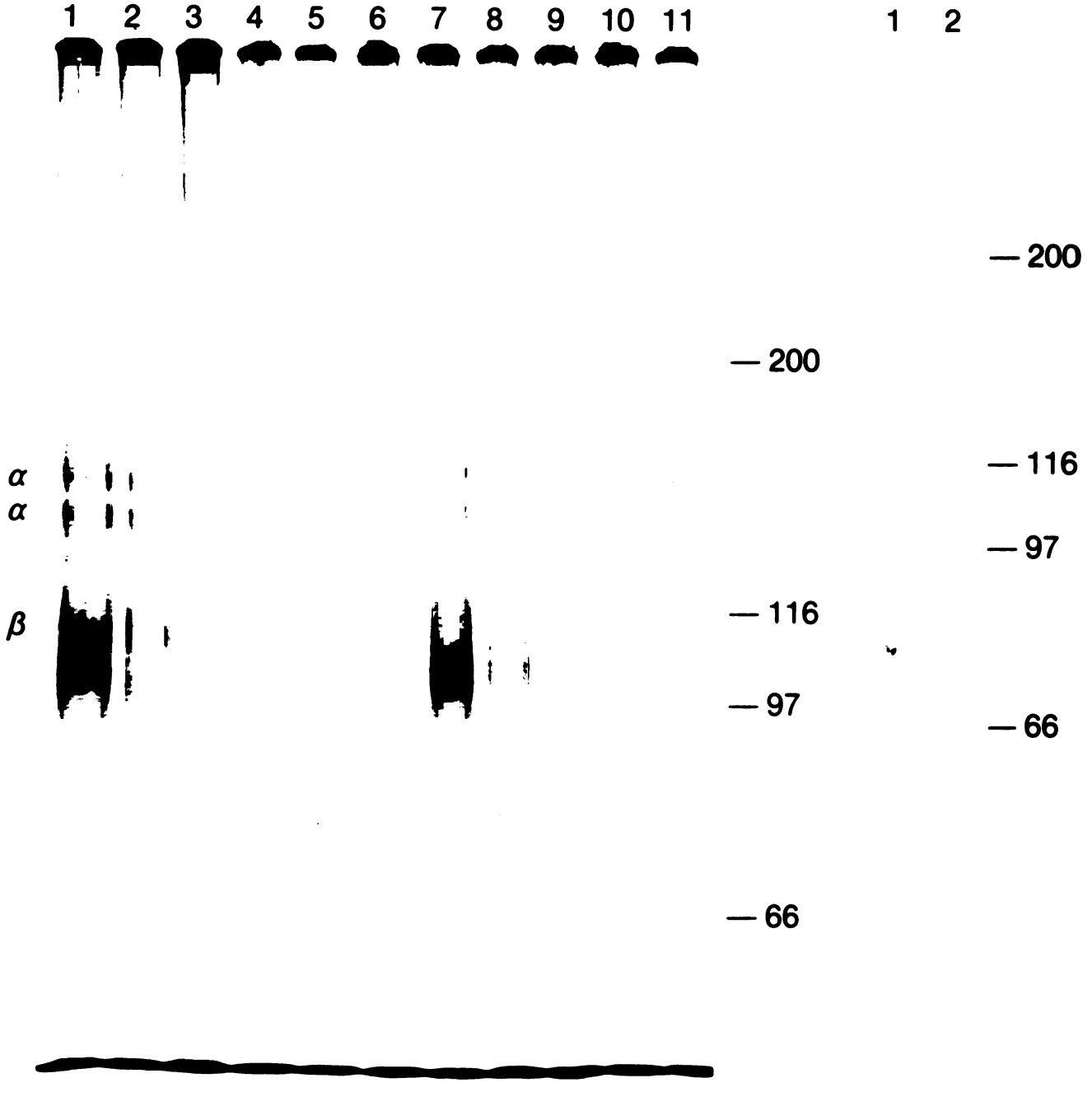
80
60
40
20
0
no Ab
GRGDSP
CSAT
TASC
TASC+CSAT
5 min
HD11 cells

Figure 4.2: TASC recognizes the integrin β_1 subunit. a. Immunodepletion analysis of the TASC antigen on E7 retinal neurons reveals that sequential precipitation of labeled extract with CSAT (lanes 1-4) removes the TASC antigen (lane 5) but not the α_v subunit (lane 6). Depletion with TASC (lanes 7-10) removes the CSAT antigen (lane 11). Both TASC and CSAT immunoprecipitate the integrin β_1 subunit of 100 and 110kD and associated α subunits at 135 and 145kD. b. Immunoblot of cell extracts with TASC shows that TASC binds a 100-110kD band corresponding to β_1 in retinal cells (lane 1) and HD11 cells (lane 2).

METHODS. E7 retinal neurons were metabolically labeled with 100 µCi/ml ^{35}S-methionine/cysteine (NEN) for 16-20 hours. Cells were mechanically removed from their plates, spun down, and extracted for 15 min in an equal volume of ice-cold Ca^{++}/Mg^{++}-containing PBS plus 1% Triton X-100 and 2mM PMSF. The supernatants were divided into 200 µl aliquots, and 20 µg of each antibody was added. Samples were rocked overnight at 4°C and then pre-cleared twice with 50 µl of Sepharose Cl-4b (Pharmacia). Antibody-antigen complexes were precipitated with Protein A-Sepharose Cl-4b and washed extensively with 0.05M Tris pH 8.0/ 150 mM NaCl/ 1mM $CaCl_2$/ 1mM $MgCl_2$/ 0.05% Triton X-100. For immunodepletion studies, the extract supernatant was repeatedly precipitated with 20 µg of the depleting antibody. An additional Protein A-Sepharose adsorption step was performed before adding the second antibody to be sure that all of the depleting antibody had been removed from the extract. The resulting supernatant was divided in half and precipitated with 20 µg of the second antibody. Pellets were extracted in sample buffer and separated by non-reducing SDS-PAGE on 6% gels. Gels were fixed, stained, treated with En^{3}Hance (NEN), dried, and exposed to Kodak X Omat R film. For immunoblots, E7 retinae and HD11 cells were extracted as above, and 200 µg of supernatant was run under non-reducing conditions on 6% gels and

transferred to nitrocellulose. Blots were incubated with 20 mg/ml TASC overnight at 4oC, followed by alkaline phosphatase-conjugated second antibody (Promega), used and developed according to Promega's specifications. Molecular weight standards were from Biorad.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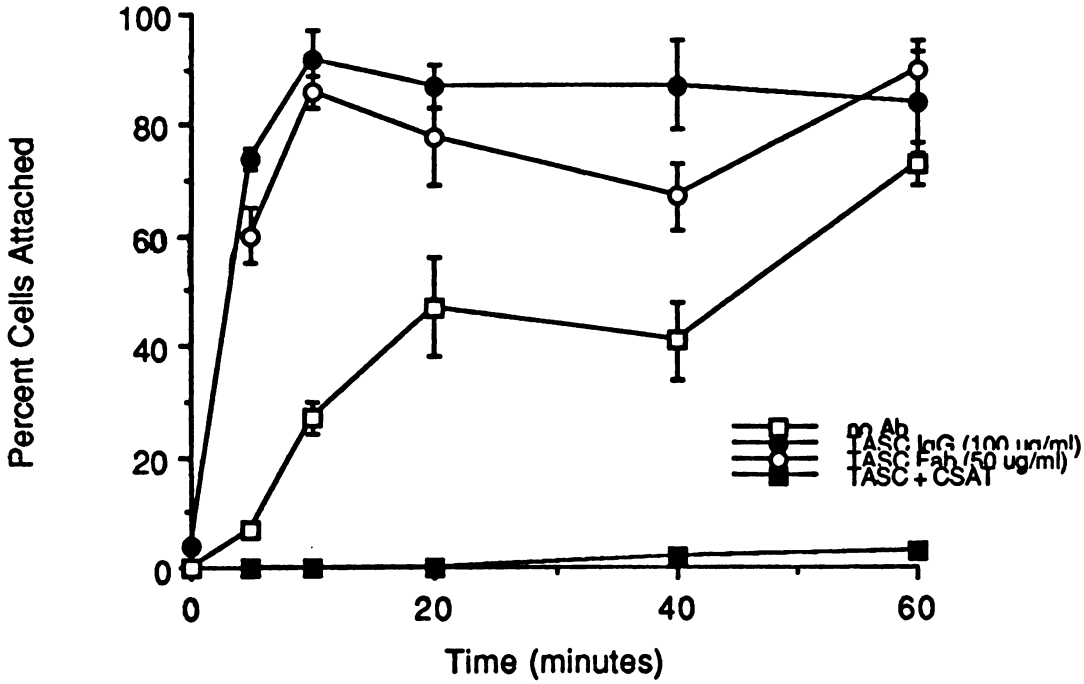
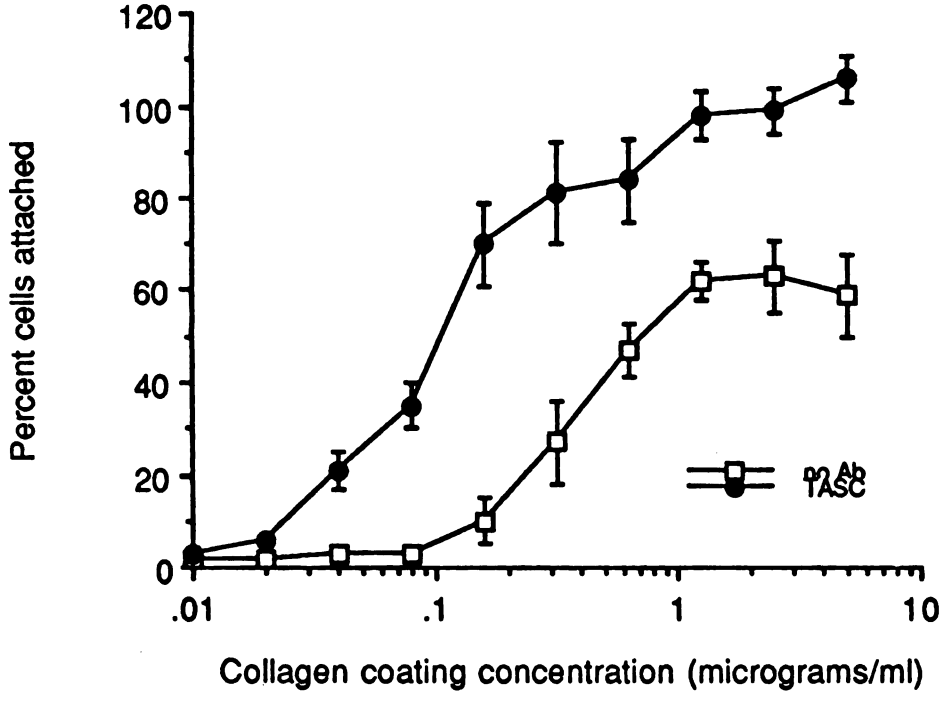
1 2
— 200
— 200
α
α
— 116
— 97
β
— 116
— 97
— 66
— 66

Figure 4.3: TASC IgG and Fab' fragments promote cell adhesion to all β_1-class integrins ligands tested. a. Time course of E7 retinal neuron attachment to Col I in the presence of no antibody, TASC IgG, TASC Fab and TASC IgG plus CSAT. b. Attachment of E7 retinal neurons to a Col I concentration curve at 40 min in the presence of no antibody or TASC at 25 μg/ml. c. Histograms of retinal neuron attachment to Col IV (10 min), LN (20 min), and FBG (1 h). d. Histograms of HD11 attachment to Col I (5 min), the 120kD cell-binding fragment of fibronectin (FN120, 0 min), and FBG (40 min). In c and d, all antibodies are used at 50 μg/ml. In a-d, all values are expressed as a percent of the attachment observed on poly-D-lysine which is considered to represent a signal of 100%.

METHODS. See Fig. 1. Col I (Collaborative Research) was diluted to 10 μg/ml in 0.1N acetic acid. LN (diluted to 20 mg/ml in CMF-PBS) was purified from murine EHS tumor in the laboratory by Drs. I. de Curtis and M.J. Ignatius according to published procedures (Timpl et al., 1979). FBG was the generous gift of Dr Z. Ruggeri, Research Institute of the Scripps Clinic (La Jolla, CA) and was used at 20 μg/ml in Ca++/Mg++-containing PBS. FN120, used at 20 μg/ml in CMF-PBS, was obtained from Calbiochem. Poly-D-lysine was from Sigma. TASC Fab fragments were generated from purified IgG by papain (Sigma) digestion followed by ion-exchange chromatography on DE-52 (Whatman).


Percent Cells Attached
100
80
60
40
20
0
0
20
40
60
Time (minutes)
mAb
TASC IgG (100 ug/ml)
TASC Fab (50 ug/ml)
TASC + CSAT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100
80
60
40
20
0
Percent cells attached
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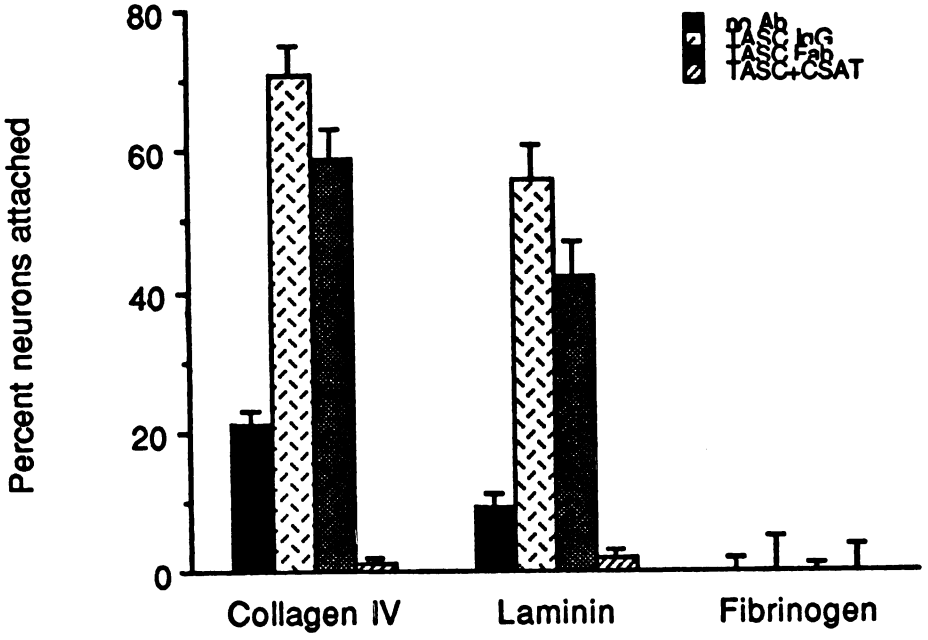
.1
1
10
Collagen coating concentration (micrograms/ml)
mAb
TASC


80
60
40
20
0
Percent neurons attached
mAb
TASC IgG
TASC Fab
TASC+CSAT
Collagen IV
Laminin
Fibrinogen

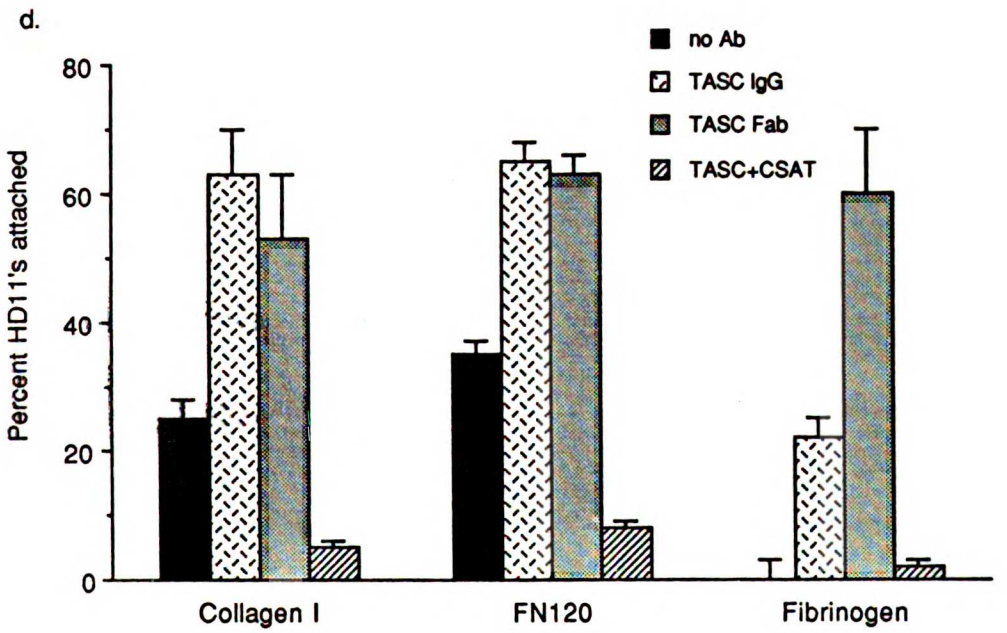

d.
no Ab
TASC IgG
TASC Fab
TASC+CSAT
Percent HD11's attached
80
60
40
20
0
Collagen I
FN120
Fibrinogen

Figure 4.4: E11 retinal neurons do not adhere to LN in the absence of TASC at any time point up to 60 min (open squares). In the presence of 100 μg/ml TASC (filled circles), attachment to LN is detectable within 10 min and peaks at 40 min with ~80% of the neurons attached compared to poly-D-lysine. TASC-induced attachment is completely blocked by the addition of CSAT (filled squares).

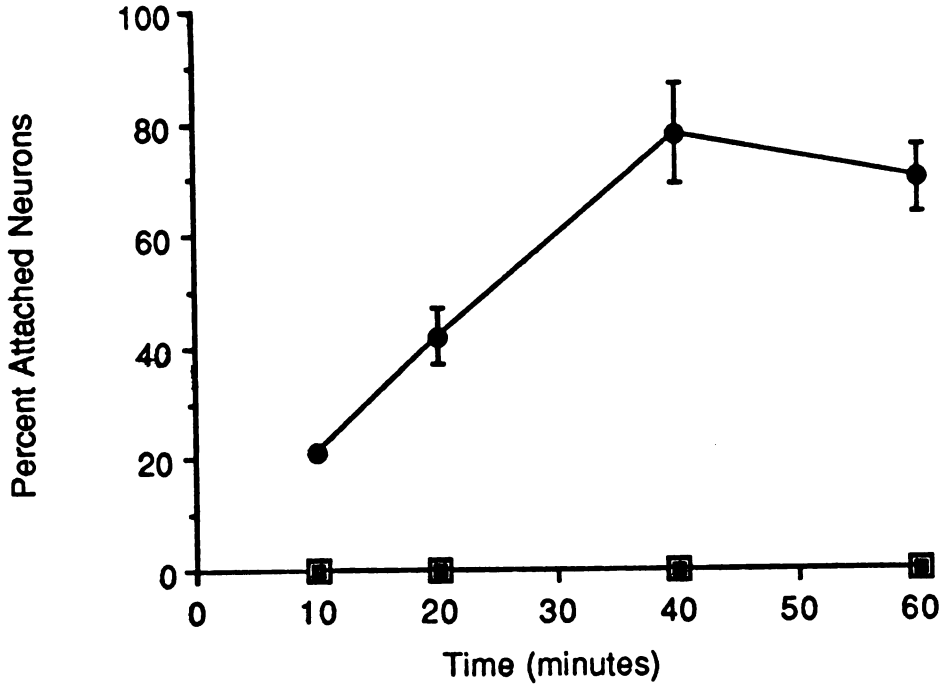
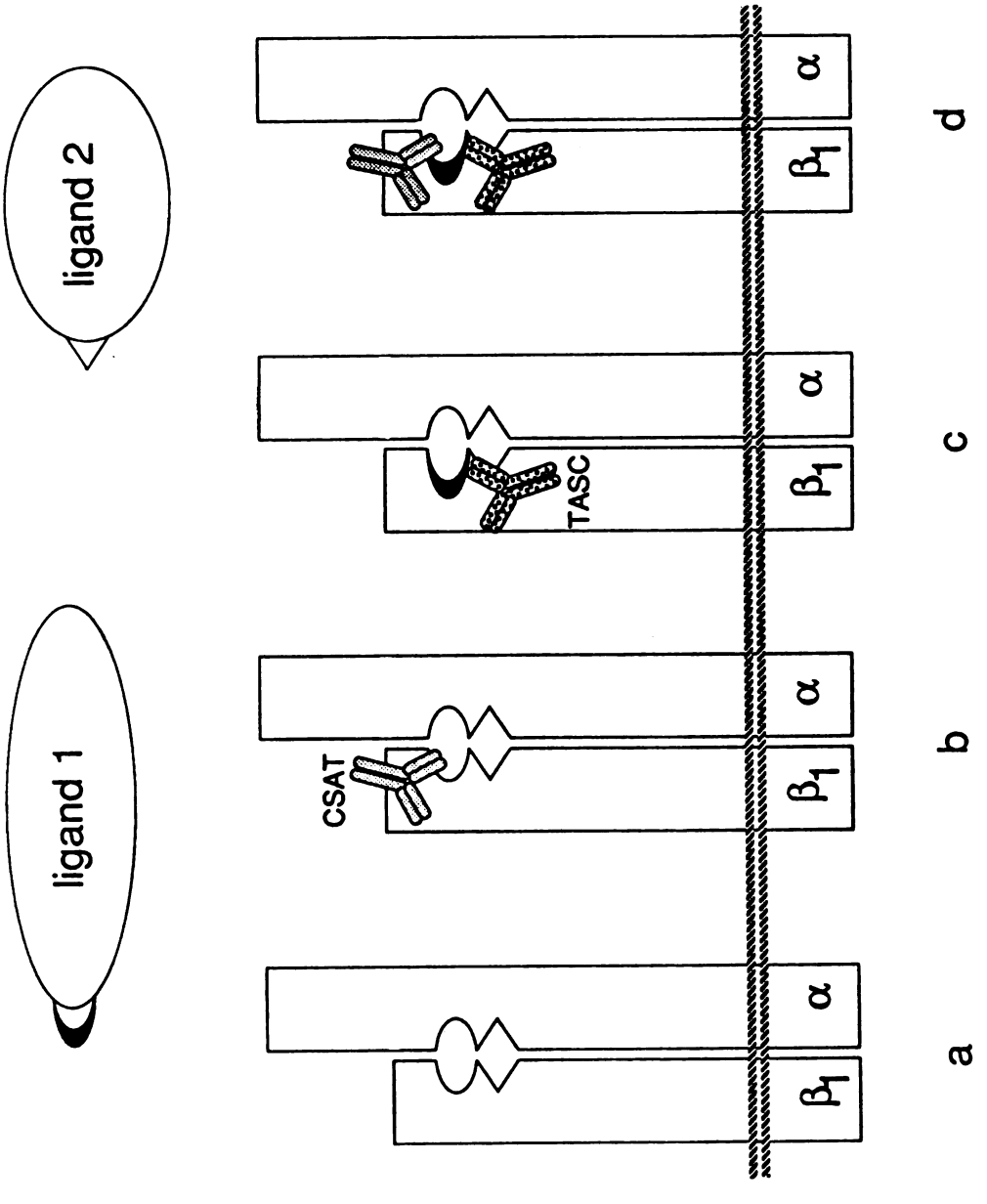

Percent Attached Neurons
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)

Figure 4.5: The effects of TASC and CSAT on cell attachment suggest a general scheme for β_1-class integrin structure and function which features two distinct sites on the β_1 subunit involved in forming ligand-binding domains as well as a conformational change that leads to higher affinity binding at one of these sites. The α subunit depicted is a composite, allowing for all of the possible ligand-binding domains formed by $\alpha\beta$ dimers, and is not meant represent any particular α subunit. a. In the absence of antibodies, β_1-class integrins bind ligands "1" (at least LN, FN120, collagens, and FBG) and "2" (VN). b. CSAT (stippled IgG) interferes with a site on the β_1 subunit required for the binding of ligand 1. c. TASC (spotted IgG) binds a distinct site on the β_1 subunit that is required for binding to ligand 2. Occupancy of this site by TASC results in an activation of the site perturbed by CSAT as indicated by shading. This site now binds ligand 1 more effectively. d. CSAT continues to recognize and perturb β_1 function in the presence of TASC, and thus even activated receptors are unable to bind ligand. Note that the antibodies are not drawn to scale but serve to indicate distinct sites on the β_1 subunit.


ligand 2
ligand 1
CSAT
TASC
α
β1
a
b
c
d

CHAPTER FIVE

Vitronectin and Thrombospondin Promote Retinal Neurite Outgrowth: Developmental Regulation and Role of Integrins

Abbreviations used in this chapter: CAM, cell-cell adhesion molecule; ECM, extracellular matrix; VN, vitronectin; TSP, thrombospondin.

SUMMARY

Protein components of basal laminae are likely to play a major role in the growth and guidance of developing axons. We have examined embryonic chick retinal neuron responses to two such molecules, vitronectin and thrombospondin, in an effort to identify new neurite outgrowth-promoting molecules and their receptors. Retinal neurons attach and extend neurites on substrates coated with either protein, and the ability to extend neurites on either is developmentally regulated. β_1- and α_v-containing integrin heterodimer function is implicated in both responses to vitronectin. On thrombospondin, integrin function is essential for neurite outgrowth, but attachment is likely to be mediated by a heparan sulfate proteoglycan(s). Vitronectin is shown to be expressed in the developing retina, implicating both proteins and their receptors in the regulation of retinal neuron process growth in vivo.

INTRODUCTION

Axonal growth and guidance is believed to depend in large part on adhesive interactions between ligands in the neuronal environment and receptors expressed on the surface of neuronal growth cones (cf. Lander, 1987; 1989; Jessell, 1988). The use of cell culture systems has greatly facilitated the identification of a number of neurite-outgrowth promoting molecules. These include neurite-promoting cell adhesion molecules (CAMs), such as N-cadherin, NCAM, and L1 (NgCAM, G4), that are expressed on the surfaces of neurons and non-neuronal cells and are likely to regulate axonal growth on cell surfaces in vivo. Secreted glycoprotein constituents of basal laminae and extracellular matrices comprise a distinct class of neurite-promoting molecules. These include laminin, fibronectin, and several collagens, each of which is expressed in extracellular spaces in developing neural tissues. Many different types of cultured neurons respond to substrates coated with these purified proteins (cf. Lander, 1987; Reichardt and Tomaselli, 1991).

The most prominent neuronal receptors for ECM constituents are members of a family of $\alpha\beta$ heterodimeric receptors called integrins. A subset of this family, the β_1-class integrins, has been shown to mediate the interactions of central neurons, peripheral neurons, and neuronal cell lines with ECM proteins like laminin (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986; Hall et al., 1987; Cohen et al., 1987; Tomaselli et al., 1987). In addition, β_1-class integrins promote neurite outgrowth on the surfaces of cultured non-neuronal cells that secrete ECM glycoproteins, in particular glial cells (Tomaselli et al, 1986; Bixby et al., 1987; Tomaselli et al., 1988b). Thus, integrins are likely to regulate the growth of axons along the pathways where their ligands are expressed.

Embryonic chick retinal neurons express a repertoire of β_1-class integrins that mediate adhesion and neurite outgrowth on laminin, collagens, fibronectin, and astroglial cell surfaces (Hall et al., 1987; Neugebauer et al., 1988). Retinal neurite

outgrowth on astroglia reflects the activity of the CAMs, N-cadherin, NCAM and L1, in addition to β_1-class integrins (Neugebauer et al., 1988; Drazba and Lemmon, 1989). However, retinal neurons continue to extend neurites on astrocytes even in the presence of combinations of function-blocking antibodies that completely inhibit neurite outgrowth by embryonic chick ciliary neurons (Neugebauer et al., 1988; Tomaselli et al., 1988b). These observations suggest that retinal neurons express receptors for additional ligands expressed by astrocytes that are not recognized by ciliary neurons. The identification of these ligands and their receptors is essential to a complete understanding of the molecular basis for axonal growth and guidance within the eye and in the optic pathway.

One possibility is that other classes of integrin heterodimers mediate neuronal responses to additional ECM molecules in the retina. For example, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ heterodimers have been shown to mediate cell adhesion to vitronectin, and $\alpha_v\beta_3$ also binds thrombospondin (Cheresh, 1987; Cheresh and Spiro, 1987; Lawler et al., 1988; Cheresh et al., 1989). If functional on neurons, neither receptor would be inhibited by integrin β_1 antibodies. In addition, there is evidence suggesting that non-integrin glycoproteins, notably platelet gpIV and a heparan sulfate proteoglycan(s), may function as receptors for thrombospondin (Asch et al., 1987; Kaesberg et al., 1989; Sun et al., 1989). Thus, integrins containing the β_3 and β_5 subunits as well as other cell surface glycoproteins are candidates for mediating retinal neurite outgrowth in response to additional ECM constituents distinct from laminin, the collagens, and fibronectin.

In the present study, we have examined neuronal responses to two prominent constituents of the ECM, vitronectin (VN) and thrombospondin (TSP), in an effort to identify new neurite-outgrowth promoting molecules. VN is present in basement membranes and extracellular spaces in human embryonic lung and the chorionic layer of fetal membranes, striated skeletal muscle, skin, and kidney (Hayman et al.,

1983). In the present study, we describe its distribution in the embryonic neural retina. TSP is synthesized by glia in vitro and is expressed in the developing mouse nervous system, including the neural retina (Asch et al., 1986; O'Shea and Dixit, 1988). Here we show that both VN and TSP promote retinal neurite outgrowth, making them likely regulators of axon growth in the embryonic nervous system. The present study also characterizes the neuronal receptors for these two neurite outgrowth-promoting molecules.

EXPERIMENTAL PROCEDURES

Adhesive Proteins

VN was purified from fetal bovine serum or chicken serum (Pelco, Rogers, AR) by chromatography on Heparin Sepharose Cl-6B (Pharmacia, Uppsala, Sweden) as described (Yatogo et al., 1988). Chicken VN was subjected to two passages of adsorbtion and elution over Heparin-Sepharose Cl-6B at the end of which a single 70kD band was visible by Coomassie blue staining of a 10% gel. This is the observed molecular weight of chicken VN purified by others (Kitagaki-Ogawa et al., 1990). Human platelet TSP was the generous gift of Dr D. Mosher, University of Wisconsin (Madison, WI).

Cell Culture

Dissociated embryonic chick retinal neurons were prepared from chick embryos (Featherhill Farms, Petaluma, CA) as described in Hall et al. (1987). For neurite outgrowth assays, 5,000 cells were plated per well (0.28 cm^2, area of the bottom of the well) of a 96-well plate that had been coated with the indicated protein. Protein-coated plastic surfaces were always blocked with 1%BSA (see below) to eliminate non-specific cell interactions with the plastic and to prevent the sticking of proteins in the medium.

Cell Attachment Assays

Cell attachment was measured as previously described in Hall et al. (1987) with the following modifications: Non-tissue culture plastic, 96-well Linbro Titertek plates (Flow Laboratories, McLean, VA) were coated with 75µl of protein-coating solution. VN and TSP were diluted to various concentrations in CMF-PBS and Ca^{++}/Mg^{++}-containing PBS (above solution plus 1mM $CaCl_2$ and 1mM $MgCl_2$), respectively. After overnight incubation at 4°C, each well was incubated with 1%

BSA in PBS for 3 hours at 37°C to eliminate non-specific binding. Antibodies and peptides were added to the wells in serum-free medium at twice the final concentration and allowed to equilibrate in the incubator while the cells were being prepared. 100,000 E7 and E11 retinal neurons were seeded in each well, and the plates were spun at 48 x g for 2 min. After incubation at 37°C of varying duration, non-adherent cells were washed from the substrate. Adherent cells were fixed overnight at 4°C in 2.5% glutaraldehyde in PBS, stained with 0.5% crystal violet, solubilized with 1% SDS, and quantitated by A_{540} (Bodary et al., 1989) in a microtiter plate reader (Flow Laboratories). In each experiment, all determinations were done in triplicate.

Antiserum to chicken vitronectin

Rabbits were immmunized with purified chicken VN by Caltag (South San Francisco, CA) and successive bleeds were tested by ELISA and immunoblot for immunoreactivity with the purified antigen. IgG was purified from serum by 50% ammonium sulfate precipitation followed by ion exchange chromatography on DEAE-cellulose (DE52; Whatman, Clifton NJ) as described by Hudson and Hay (1980).

Immunoblotting

Extracts of embryonic chicken neural retinae, vitreous humour, and lens (all avascular tissues) were prepared by careful dissection in CMF-PBS to avoid contamination with blood. Vitreous humours were extracted directly in sample buffer. Retinae and lenses were pelleted and resuspended in 1% Triton X-100 (Sigma, St. Louis, MO) in PBS. After extraction on ice for 15 minutes, extracts were spun for 15 minutes in a refrigerated microfuge and pellets were discarded. Tissue extracts (50-200 µg/lane) were run under reducing conditions on 10%

polyacrylamide gels by SDS-PAGE. After transfer to 0.2 μm nitrocellulose, blots were blocked with blotto: 5% nonfat dry milk (Carnation) in 50mM Tris-HCl pH7.5/ 150mM NaCl. Blots were incubated with 20 μg/ml anti-VN IgG or preimmune serum at a 1:100 dilution in blotto overnight at 4°C. After washing in blotto, incubation in alkaline phosphatase-conjugated anti-rabbit second antibody (Promega, Madison, WI) and further washing, alkaline phosphatase reaction product was generated by developing at pH 9.5 with NBT and BCIP (Sigma).

Immunostaining

Heads of E6 chicken embryos were embedded in polyester wax according to Sheppard et al. (1988). Briefly, the heads were washed in PBS and fixed for 6h at 4°C in 5% glacial acetic acid in ethanol. They were washed in 100% ethanol for 1h at 37°C and then impregnated with polyester wax over a graduated series of 3:1, 2:1, 1:1 mixtures with 100% ethanol 1h each at 37°C. After overnight incubation in the wax, the tissue was embedded in fresh wax, left to harden at -20°C, and sectioned at 6 μm in the sagittal plane. Sections were placed on 0.2% gelatin-coated slides, dried, dewaxed in 100% ethanol and rehydrated in a graded series. Sections were blocked in 5% normal goat serum (NGS) in Tris-buffered saline pH 7.4 (TBS) for 30 min at room temperature. Rabbit anti-chicken VN IgG was diluted to 10 μg/ml and preimmune serum to 1:500 in NGS/TBS. Sections were incubated with either antibody for 1h at room temperature, washed 3 times in NGS/TBS for 15 min before using the Vectastain ABC kit (Vector Labs, Inc.) for detection by HRP reaction product. The sections were incubated with biotinylated secondary antibody for 30 min at RT, washed 3 times in NGS/TBS, incubated in HRP-avidin for 30 min at RT, and washed 3 times. Peroxidase was reacted with diaminobenzidine tetrahydrochloride (500 μg/ml, pH5.6) for 5 min at RT.

Monoclonal antibodies

Hybridoma cells secreting the CSAT monoclonal antibody were the generous gift of Dr A.F. Horwitz, University of Illinois (Urbana, IL). LM609 ascites fluid was kindly provided by Dr D.A. Cheresh, Research Institute of the Scripps Clinic (La Jolla, CA). The TASC and Chav-1 monoclonal antibodies were isolated in this laboratory, using E10 chick brain glial cells as the immunogen and following the protocol of Harlow and Lane (1988). Spleen cells were fused with the SP2-0 hybridoma cell line. Clonal supernatants were screened for their ability to inhibit retinal neuron adhesion to VN, using the quantitative attachment assay described below. Upon their identification as blocking antibodies, TASC and Chav-1 were subcloned by limiting dilution, and secreting clones were selected. Both antibodies were found to be IgG_1's, using a subtyping kit from Calbiochem (San Diego, CA).

CSAT, LM609, Chav-1 and TASC IgG's were purified from ascites fluid by 50% ammonium sulfate precipitation, followed by chromatography on Protein A-Sepharose Cl-4B (Pharmacia, Uppsala, Sweden) as per Ey, et al. (1978). IgG's were dialyzed against calcium/magnesium-free PBS (CMF-PBS; 200 mg/l KCl, 200mg/l K_2SO_4, 8 g/l NaCl, and 2.16 g/l $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) and stored at -70°C.

Immunoprecipitation

E7 retinal neurons were metabolically labeled with 100 mCi/ml ^{35}S-methionine/cysteine (NEN, Boston, MA) for 16-20 hours. Cells were removed from their plates by gentle pipetting, spun down, and resuspended in and equal volume of ice-cold Ca^{++}/Mg^{++}-containing PBS plus 1% Triton X-100 and 2mM PMSF. After 15 min of incubation on ice, the extracts were spun at 10,000 rpm in a refrigerated microfuge. The supernatants were divided into 200µl aliquots, and 20 µg of each antibody was added. Samples were rocked overnight at 4°C and then pre-cleared twice with 50 µl of Sepharose Cl-4b (Pharmacia). Antibody-antigen complexes

were precipitated with Protein A-Sepharose Cl-4B (Pharmacia) and washed extensively with 0.05M Tris pH 8.0/ 150 mM NaCl/ 1mM $CaCl_2$/ 1mM $MgCl_2$/ 0.05% Triton X-100. For immunodepletion studies, the extract supernatant was repeatedly precipitated with 20 μg of the depleting antibody. An additional Protein A-Sepharose adsorption step was performed before adding the second antibody to be sure that all of the depleting antibody had been removed from the extract. The resulting supernatant was divided in half and precipitated with 20 μg of the second or test antibodies. Pellets were extracted in sample buffer and separated by SDS-PAGE on 6% gels. Gels were fixed, stained, treated with En^{3}Hance (NEN), dried, and exposed to Kodak X Omat AR film (Rochester, NY). Molecular weight standards were from Biorad (Richmond, CA).

Immunoblots of immunoprecipitated material were performed by precipitating unlabeled extracts of 10 E7 retinae each with the indicated antibodies. Antibody-antigen complexes were run under reducing conditions on 6% gels so that the reduced antibody bands would not run in the same molecular weight range as the antigens. Nitrocellulose blots of these gels were incubated with polyclonal antisera raised to either the chicken β_1 subunit (Damsky et al., 1985) or the extracellular domain of the chicken α_v subunit (Bossy and Reichardt, 1990) as described above.

RESULTS

Embryonic chick retinal neurons adhere and extend neurites on vitronectin and thrombospondin

To assess possible roles of additional ECM constituents in mediating axon growth, we examined neuronal responses to purified VN and TSP. Figure 5.1 shows that dissociated embryonic day 7 (E7) retinal neurons extended neurites on substrates coated with either glycoprotein after 24 hours of culture. In this experiment, 39+/-3% of the neurons on VN and 34+/-5% on TSP bore neurites longer than two cell bodies. With respect to this parameter of neurite outgrowth, therefore, VN and TSP stimulate a response comparable to that seen on astrocyte monolayers (Neugebauer et al., 1988). In contrast, embryonic day 11 (E11) retinal neurons remained round when cultured on either VN or TSP and did not extend neurites longer than two cell bodies (Figure 5.1). However, these older neurons were not clumped or floating when cultured on either substrate, suggesting that they continue to adhere to both substrates.

To quantitatively separate neurite outgrowth-promoting activity from adhesion to VN and TSP, retinal neuron attachment was measured in short-term assays. Figure 5.2a shows that in one hour, 100% of E7 retinal neurons attached to VN with a half-maximal coating concentration of 0.2 μg/ml. ~60% of E11 retinal neurons attached to VN with the same concentration dependence as E7 retinal neurons, suggesting that 40% of the cells do not express functional VN receptors. In response to TSP (Figure 5.2b), ~80% of E7 and ~40% of E11 retinal neurons attached in 90 minutes with a half-maximal coating concentration of 20 μg/ml, again suggesting that the expression and/or activity of receptors for TSP is developmentally regulated.

Vitronectin is expressed in the embryonic neural retina

Results presented above establish both VN and TSP as potential regulators of neuronal development in the embryonic neural retina. Consistent with this possibility, O'Shea and Dixit (1988) have detected TSP expression in the developing murine neural retina. To determine whether VN is expressed in the chicken neural retina, chicken VN was purified from serum (Figure 5.3, lane 1) and used to prepare a specific rabbit antiserum. The IgG fraction was shown to abolish cell adhesion to purified chicken VN (data not shown). Figure 5.3 shows that immunoreactive proteins migrating with a similar Mr (a 74/70k doublet) to purified chicken VN are present in extracts of E7 and E11 neural retinae. Since the chicken retina is avascular, these proteins are not likely to reflect VN derived from blood. In the E7 lens and vitreous humour, a similar band with an Mr of 70k was also detected (lanes 4 and 5).

Anti-chicken VN IgG was used to stain sections optic tissue. VN immunoreactivity was prominent in the E5 retina, optic stalk and nerve (Figure 5.4a). At E6, VN was detected in the extracellular spaces of the neural retina where reaction product outlined the profiles of cell bodies. The strongest staining was observed at the basement membrane adjacent to the optic fiber layer (Figure 5.4b). The lens and vitreous humour were also reactive (data not shown), consistent with the results from immunoblots. At E12, VN staining was most intense in the optic fiber layer and in the inner and outer plexiform layers. The cell bodies of the ganglion cells in particular were outlined by reaction product (Figure 5.4d). Thus, VN is expressed in the developing chicken neural retina in positions consistent with its interaction with both cell bodies and neural processes.

Neuronal receptors for vitronectin and thrombospondin

Integrin dimers containing the α_v subunit in association with either β_1, β_3, or β_5

have each been shown to mediate cell adhesion to VN (Cheresh, 1987; Cheresh and Spiro, 1987; Cheresh et al., 1989; Bodary and McLean, 1990). These interactions are inhibited by peptides containing arg-gly-asp (RGD), a sequence derived from the only identified cell attachment site in VN. In initial experiments, the RGDS peptide was shown to block E7 retinal neuron attachment to VN (chapter 4; see Figure 5.7a) and to almost completely eliminate neurite outgrowth on VN (Table 5.1), suggesting that RGD-sensitive integrins function as neuronal VN receptors. To identify these receptors, we initiated a monoclonal antibody (mAb) screen, testing clonal supernatants for their ability to decrease retinal neuron attachment to VN in our quantitative assay.

Of 700 clones, two secreted antibodies that reproducibly inhibited attachment to VN. One of these, named TASC, recognizes a novel epitope on the integrin β_1 subunit (chapter 4), establishing a role for an integrin β_1 heterodimer(s) in neuronal responses to VN. A second mAb, named Chav-1, also inhibited E7 retinal neuron attachment to VN in a manner that was additive with TASC (Figure 5.5). The relative effects of both antibodies were greater at earlier times in the process of cell adhesion, but even the combination of TASC plus Chav-1 did not completely inhibit attachment to VN (Figure 5.5). Essentially identical results were obtained with E11 retinal neurons (data not shown). These data suggest that VN receptors not recognized by either antibody might also be present on the neuronal cell surface.

To characterize the Chav-1 antigen biochemically, metabolically labeled E7 retinal neurons were immunoprecipitated with Chav-1 IgG. Three proteins were specifically precipitated by Chav-1, a major band of Mr 140k, and two bands of lower intensity at 100 and 92kD (Figure 5.6a, lane 1). Further studies suggest that Chav-1 recognizes the integrin α_v subunit at 140kD. We used the complex-dependent $\alpha_v\beta_3$ mAb, LM609 (Cheresh, 1987) to show that E7 retinal neurons

synthesize $\alpha_v\beta_3$ heterodimers, migrating at 140 (α_v) and 92kD (β_3) (lane 7). Chav-1 depletes retinal extracts of $\alpha_v\beta_3$ heterodimers, since $\alpha_v\beta_3$ is not detected upon subsequent precipitation with LM609 (lane 6). TASC precipitated a characteristic panel of β_1-class integrins from an aliquot of the same Chav-1-depleted sample (lane 5; Hall et al., 1987), showing that proteins recognized by Chav-1 are distinct from β_1-class integrin heterodimers and, furthermore, that significant glycoprotein degradation had not occurred during the course of the experiment. Repeated precipitation with LM609 removes the 92kD integrin β_3 subunit from Chav-1 immunoprecipitable material, but the 140/100kD bands persist in the LM609-depleted extract (lane 11). The 100kD β subunit is distinct from β_3, since LM609 does not recognize the dimer it forms with α_v and since a polyclonal antibody against the β_3 cytoplasmic domain precipitates only the 140/92kD heterodimer (lane 12). Thus, Chav-1 recognizes an epitope common to two integrin heterodimers, $\alpha_v\beta_3$ and α_v in association with a 100kD integrin β subunit distinct from β_3. The most likely antigenic site to be shared by these two heterodimers is the α_v subunit itself.

In addition to β_3, the α_v subunit has been shown to associate with the integrin subunits β_1 and β_5 (Cheresh et al., 1989; Bodary and McLean, 1990; Vogel et al., 1990; Bossy and Reichardt, 1990). To determine whether the α_v-associated 100kD band on E7 retinal neurons is antigenically related to the β_1 subunit, extracts of E7 retinae were immunoprecipitated with two monoclonal antibodies against β_1 (CSAT and TASC), in addition to Chav-1, and the $\alpha_v\beta_3$-specific mAb LM609. Immunoprecipitated material was separated under reducing conditions by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with polyclonal antibodies against either the chicken β_1 subunit (Damsky et al., 1985) or a peptide from the extracellular domain of the chicken α_v subunit (Bossy and Reichardt, 1990). Figure 5.6b shows that the β_1 subunit was detected in the material precipitated by TASC

but not by Chav-1. As expected, no β_1 reactivity was present in the LM609 precipitate which should only contain the α_v and β_3 subunits. In the converse experiment, the α_v subunit was detected in material precipitated by Chav-1 but not by CSAT or TASC (Figure 5.6b). These experiments show that α_v does not associate with β_1 on retinal neurons to produce VN-binding heterodimers, consistent with the observation that the effects of TASC and Chav-1 are additive and, thus, likely to perturb the function of distinct integrin heterodimers. Since the 100kD protein is antigenically unrelated to β_1 and β_3, it appears to represent an additional β subunit, most likely β_5 (Cheresh et al., 1989).

Since a number of potential VN receptors appear to be expressed by retinal neurons, the effects of antibody combinations on attachment to VN were measured. Figure 5.7a shows that TASC and Chav-1 inhibit retinal neuron attachment to VN alone and in combination. The anti-integrin β_1 mAb CSAT recognizes an epitope distinct from TASC's (chapter 4) and was less effective than TASC in reducing neuronal attachment to VN. The mAb against $\alpha_v\beta_3$ (LM609) did not inhibit VN attachment and did not potentiate the effects of TASC, Chav-1, or CSAT. LM609 is known to block the function of chicken $\alpha_v\beta_3$, since it inhibited the attachment of the chicken HD11 myeloblast cell line to VN and fibrinogen (see appendix B). Thus, these results do not support a role for $\alpha_v\beta_3$ in mediating retinal neuron interactions with VN. Instead, retinal neuron attachment to VN appears to be mediated by at least two integrin heterodimers: one composed of α_v in association with a 100kD β subunit and another composed of β_1 in association with an unknown α subunit.

On endothelial cells, the integrin heterodimer $\alpha_v\beta_3$ has been implicated in arg-gly-asp-dependent interactions with TSP (Lawler et al., 1988). To determine whether neuronal integrins mediate responses to TSP, we assayed the effects of the GRGDSP peptide and the function-blocking antibodies, CSAT, TASC, Chav-1, and

LM609. Neither the peptide nor any of the antibodies alone or in combination decreased neuronal attachment to TSP (Figure 5.7b). Thus, it seemed unlikely that neuronal cell attachment to TSP was mediated by integrins containing either the β_1 or α_v subunits.

To explore the possibility that a heparan sulfate proteoglycan(s) plays a role in neuronal interactions with TSP, the effects of soluble heparin on attachment to TSP and VN were measured. Figure 5.7c shows that heparin completely blocked neuronal attachment to TSP with half-maximal activity at 0.2 μg/ml which is equivalent to 0.035 U/ml. This concentration-dependence of inhibition is in agreement with previously published studies using CHO cells (Kaesberg et al., 1989). Preincubation of the TSP substrate with 100 μg/ml heparin also abolished attachment, indicating that heparin elicits its effects by binding to TSP and not to the neuronal surface (data not shown). Neuronal attachment to VN was not inhibited by heparin in a parallel experiment (Figure 5.7c).

To extend our findings on attachment to retinal neurite outgrowth, we measured the effects of the same panel of reagents on the extent of E7 retinal neurite outgrowth on VN and TSP. Results presented in Table 5.1 show that 30+/-2% of the neurons extended neurites on VN in the absence of function-blocking reagents. The GRGDSP peptide dramatically decreased the percentage of neurons with neurites to 6%, consistent with its complete inhibition of neuronal attachment to VN (Figure 5.7a). The greatest effect of any of the mAbs was seen with Chav-1 which reduced the percent of neurite-bearing neurons to 15% when added alone. The anti-β_1 mAb CSAT had significant, though weaker, inhibitory effects when added alone, and did not dramatically potentiate the effects of Chav-1 when the two antibodies were added together. The inability of the combination of CSAT plus Chav-1 to completely abolish neurite outgrowth on VN, suggests that other neuronal VN receptors may also mediate neurite outgrowth in response to this ligand.

However, neurite outgrowth was not perturbed by the addition of either the anti-β_1 mAb TASC or the anti-$\alpha_v\beta_3$ mAb, LM609. These results suggest that the α_v/100kβ heterodimer is the major neurite outgrowth-promoting receptor that binds VN, and that a CSAT-sensitive β_1-class integrin dimer also plays a role.

The percent of neurons with neurites on TSP (34+/-5% in the control) was not reduced by the GRGDSP peptide or the anti-$\alpha_v\beta_3$ mAb LM609 (Table 5.1). Surprisingly, both anti-β_1 mAb's CSAT and TASC significantly inhibited neurite outgrowth on TSP, reducing the percent of neurite-bearing cells to 7% and 25% respectively. Chav-1 also had partial effects, reducing this value to 24%. The combination of CSAT plus Chav-1 virtually eliminated neurite outgrowth on TSP. These results, together with the attachment data in Figure 5.7b, argue that although β_1- and α_v-containing integrins do not play a dominant role in neuronal adhesion to TSP, their activity is required for TSP-stimulated neurite outgrowth.

DISCUSSION

Embryonic chicken retinal neuron responses to two secreted glycoproteins, VN and TSP, have been examined in an effort to identify neurite outgrowth-promoting molecules that may stimulate process growth in the developing optic pathway. Our results support the following conclusions: (1) Both VN and TSP promote attachment and neurite extension by early embryonic retinal neurons. (2) VN is expressed in the developing neural retina in positions consistent with its role as an adhesion and neurite-promoting molecule in vivo. (3) α_v and β_1-containing integrin heterodimers (but not $\alpha_v\beta_1$) promote attachment to VN, and the α_v-containing dimer plays the major role in VN-mediated neurite outgrowth. (4) A heparan sulfate proteoglycan(s) is likely to mediate retinal neuron attachment to TSP, while integrin function is essential to TSP-stimulated neurite outgrowth. (5) Attachment and neurite outgrowth in response to VN and TSP depend on the developmental age of the neurons, suggesting that the expression and/or activities of VN and TSP receptors are developmentally regulated.

Substrate-bound VN is known to promote the adhesion and spreading of a variety of non-neuronal cell types in vitro (Barnes et al., 1980; Cheresh and Spiro, 1987), and VN has been detected in some basement membranes (Hayman et al., 1983). The demonstration that developing retinal neurons attach and extend neurites on VN suggests that VN may play a significant role in the development of the nervous system. This possibility is substantiated by the observed expression of VN in the developing neural retina (see Figure 5.4). In the early retina (E5-6), VN immunoreactivity was detected in the optic stalk and nerve, in the inner limiting membrane (the basement membrane adjacent to the optic fiber layer) and surrounding neuroepithelial and ganglion cells. During axon growth within the eye, the growth cones of retinal ganglion cell axons are closely associated with the inner limiting membrane (Easter et al., 1984). After the formation of cellular layers (E12),

VN was detected around cell bodies and in the plexiform layers of neuronal processes. Thus, within the retina, VN is appropriately situated for interactions with cell bodies, axons, and dendrites at both stages of development. In contrast, laminin is prominent in the optic fiber layer and neuroepithelium within the very early retina (E3) but becomes restricted to the inner limiting membrane by E8 (Cohen et al., 1987). VN and its receptors are, therefore, likely regulators of events occurring during this time span of retinal development, including cell migration, cell adhesion, process outgrowth, and synapse formation.

In an effort to identify and characterize neuronal VN receptors, we generated two monoclonal antibodies, each of which partially inhibits E7 retinal neuron attachment to VN. Biochemical studies of their antigens demonstrate that one mAb, TASC, binds the integrin β_1 subunit (chapter 4) while the second, Chav-1, almost certainly recognizes the integrin α_v subunit. Two α_v-containing integrin heterodimers are expressed by E7 retinal neurons, $\alpha_v\beta_3$ and α_v in association with an unknown 100kD β subunit. However, an $\alpha_v\beta_3$ complex-specific mAb, LM609 (Cheresh, 1987), did not detectably inhibit attachment or neurite outgrowth on VN, indicating that the $\alpha_v\beta_3$ heterodimer is not an important VN receptor on these cells. The LM609 mAb does bind the chicken $\alpha_v\beta_3$ complex (see Figure 5.6), and it inhibits the interactions of chicken myeloid cells with VN (see appendix B). Retinal neurons do not respond to another $\alpha_v\beta_3$ ligand, fibrinogen, and the $\alpha_v\beta_3$-specific mAb LM609 does not inhibit retinal neuron responses to TSP, a ligand for $\alpha_v\beta_3$ on endothelial cells (Cheresh, 1987; Cheresh and Spiro, 1987; Lawler et al., 1988; chapter 4). Thus, although $\alpha_v\beta_3$ is clearly synthesized by retinal neurons, its function remains elusive. Instead, the inhibitory effects of the α_v-specific mAb Chav-1 on retinal neuron interactions with VN must reflect the function of the α_v/100kD heterodimer. This 100kD protein is not immunologically related to the integrin β_1 or β_3 subunits (Figure 6). The β_5 (β_s) or β_6 subunits are candidates for

this 100kD β subunit (Cheresh et al., 1989; Freed et al., 1989; Sheppard et al., 1990). It is interesting that α_v does not associate with β_1 on retinal neurons, since it has been shown to do so on other cell types (Bodary and McLean, 1990; Vogel et al., 1990; Bossy and Reichardt, 1990).

The isolation of an integrin β_1 subunit-specific mAb (TASC) as an inhibitor of VN-mediated attachment was surprising, since another integrin β_1 subunit-specific mAb against a distinct β_1 epitope (CSAT) blocks β_1-class integrin binding to collagens, laminin, and fibronectin, but had little effect on attachment to VN (Figure 5.7, this report; Hall et al., 1987). The fact that TASC significantly inhibits retinal neuron attachment to VN suggests that a β_1-class integrin heterodimer acts as a VN receptor on these cells. Consistent with this, others have observed VN binding to β_1-class integrins (Buck and Horwitz, 1987). TASC's activity further diverges from CSAT's, since TASC has been shown to promote rather than to inhibit integrin β_1-dependent cell attachment to collagens, laminin, and fibronectin (chapter 4). We favor the interpretation that TASC preferentially obstructs the VN-binding domain on at least one $\alpha\beta_1$ heterodimer while it activates other β_1-class dimers that binds the other ligands. Interestingly, CSAT had stronger effects on VN-stimulated neurite outgrowth than did TASC, implicating the function of a CSAT-sensitive β_1-class integrin in the neurite outgrowth response to VN. The β_1-associated α subunit(s) that produces binding is unknown, but is not likely to be α_v.

Since TSP is expressed in the developing spinal cord, dorsal root ganglion, and cerebral and cerebellar cortices and plays a role in granule cell migration in the cerebellum (O'Shea and Dixit, 1988; O'Shea et al., 1990), it is of particular interest to identify neuronal TSP receptors and to determine their cellular activities. Whereas members of the integrin family of receptors played a major role in the attachment of retinal neurons to VN, our results suggest that they are much less important in mediating attachment to TSP. None of the reagents available for

perturbing integrin function, including an RGD-containing peptide and function-blocking antibodies against the integrins $\alpha_v\beta_3$, β_1, or α_v, were able to inhibit the attachment-promoting activity of TSP. Instead, soluble heparin completely inhibited attachment to TSP, suggesting that the heparin-binding domain of TSP is the active site for retinal neuron attachment. Considerable evidence exists for the role of heparan sulfate proteoglycans and perhaps sulfated glycolipids in the attachment and spreading of non-neuronal cells on TSP (Murphy-Ullrich and Mosher, 1987; Roberts, 1988; Kaesberg et al., 1989; Taraboletti et al., 1987; 1990; Sun et al., 1989). Several transmembrane or membrane-associated heparan sulfate proteoglycans have been identified and implicated in cell interactions with the ECM, providing precedence for heparan sulfate proteoglycan's that can mediate signals involved in adhesion, spreading, and motility (Woods et al., 1985; Saunders and Bernfield, 1988). One transmembrane heparan sulfate proteoglycan, syndecan, has been shown to bind TSP, in addition to fibronectin and some collagens (Saunders and Bernfield, 1988; Sun et al., 1989). Syndecan mRNA is present in the adult brain, and it will be interesting to determine its location in the developing brain where proteoglycans are abundant and dynamically expressed (Saunders et al., 1989; Herndon and Lander, 1990).

In contrast to our results implicating heparan sulfate proteoglycans as potential receptors that mediate neuronal attachment to TSP, we found that anti-integrin antibodies inhibited E7 retinal neurite outgrowth on TSP. In particular, the anti-integrin β_1 subunit mAb, CSAT, reduced the percent of cells bearing neurites on TSP from 34% to 7%, and the α_v-specific mAb Chav-1 also had significant inhibitory effects (see Table 5.1). Thus, although integrins are not required for the attachment of cells to TSP, their activity is essential to the neurite outgrowth response. The identities of the particular $\alpha\beta$ integrin heterodimers that transduce this response are as yet unknown. Since the percentage of neurons extending

neurites on TSP was not reduced by an RGD-containing peptide, the TSP-binding integrins in question may recognize a distinct site in the TSP molecule. Consistent with this, neurite outgrowth by rat sympathetic ganglion neurons on TSP is inhibited by a mAb to the "stalk" region of TSP which is structurally distinct from the heparin-binding and RGD-containing domains (Osterhout and Higgins, 1990).

Two interesting results of the present work concern the developmental regulation of receptor activity and the relationship of receptor-mediated adhesion to neurite outgrowth. First, retinal neuron responses to VN and TSP varied with the developmental age of the neurons. While E7 neurons attached and extended neurites on both substrates, fewer E11 retinal neurons attached and none extended neurites on either substrate. Similarly, on collagen IV, E6 retinal neurons attach and extend neurites, but E11 neurons attach without extending neurites (Hall et al., 1987). Several possibilities might explain these phenomena, including: (i) A threshold level of receptor expression is required for neurite outgrowth, and this level exceeds that sufficient to produce cell adhesion. (ii) Intracellular signalling mechanisms are regulated such that adhesion and neurite outgrowth-promoting signals across the plasma membrane are distinct and, therefore, separable.

Adhesion and neurite outgrowth responses to TSP are separable, since attachment is integrin-independent and neurite outgrowth is integrin-dependent. Similarly, responses to VN are partially separable, since the mAb against α_V was more effective in reducing neurite outgrowth on VN than was the mAb against the β_1 subunit (see Table I). Nevertheless, each was equally potent as an inhibitor of neuronal adhesion to VN. Thus, for the same cell responding to a single ligand, differential receptor activities can be distinguished. Together, these observations argue that while cell adhesion is a prerequisite for neurite outgrowth in vitro, it is not the only requirement. Indeed, Gundersen (1987) has demonstrated, using a patterned laminin/collagen substrate, that growth cones prefer to grow on laminin to

which they appear to adhere less strongly. The regulation of second messenger systems is a potential site for the observed distinction between integrin-mediated adhesion and neurite outgrowth (Bixby, 1989). The identification of neuronal receptors that promote both responses to defined ligands provides an avenue for investigating this issue.

Table 5.1: The effects of the GRGDSP peptide and function-blocking monoclonal antibodies on E7 retinal neurite outgrowth. Neurons were seeded onto wells coated with 10 μg/ml VN or 50 μg/ml TSP and cultured for 24 hours in the presence of 100 μg/ml GRGDSP, 50 μg/ml CSAT, 100 μg/ml Chav-1, 200 μg/ml TASC, or 100 μg/ml LM609. After fixation, cells were scored for the presence of a neurite(s) longer than two cell bodies. A minimum of 100 neurons per well was counted, and each experiment was done in triplicate. Each value represents the mean +/- standard deviation for three separate experiments on VN and one representative experiment on TSP.

	Percent E7 Retinal Neurons with Neurites	
	VN	TSP
Control	30+/-2	34+/-5
GRGDSP	6+/-3	35+/-6
CSAT	22+/-2	7+/-1
Chav-1	15+/-6	24+/-5
CSAT+Chav-1	12+/-6	1+/-1
TASC	32+/-7	25+/-1
LM609	32+/-5	32+/-0

Figure 5.1: Retinal neuron responses to VN and TSP substrates after 24 hours of culture. E7 retinal neurons extended neurites on 10 μg/ml chicken VN (*a*) and 40 μg/ml TSP (*b*). E11 retinal neurons appeared attached to VN (*c*) and TSP (*d*) but remained round and did not extend neurites. Photographs were taken with phase contrast optics. Scale bar, 10 μm.

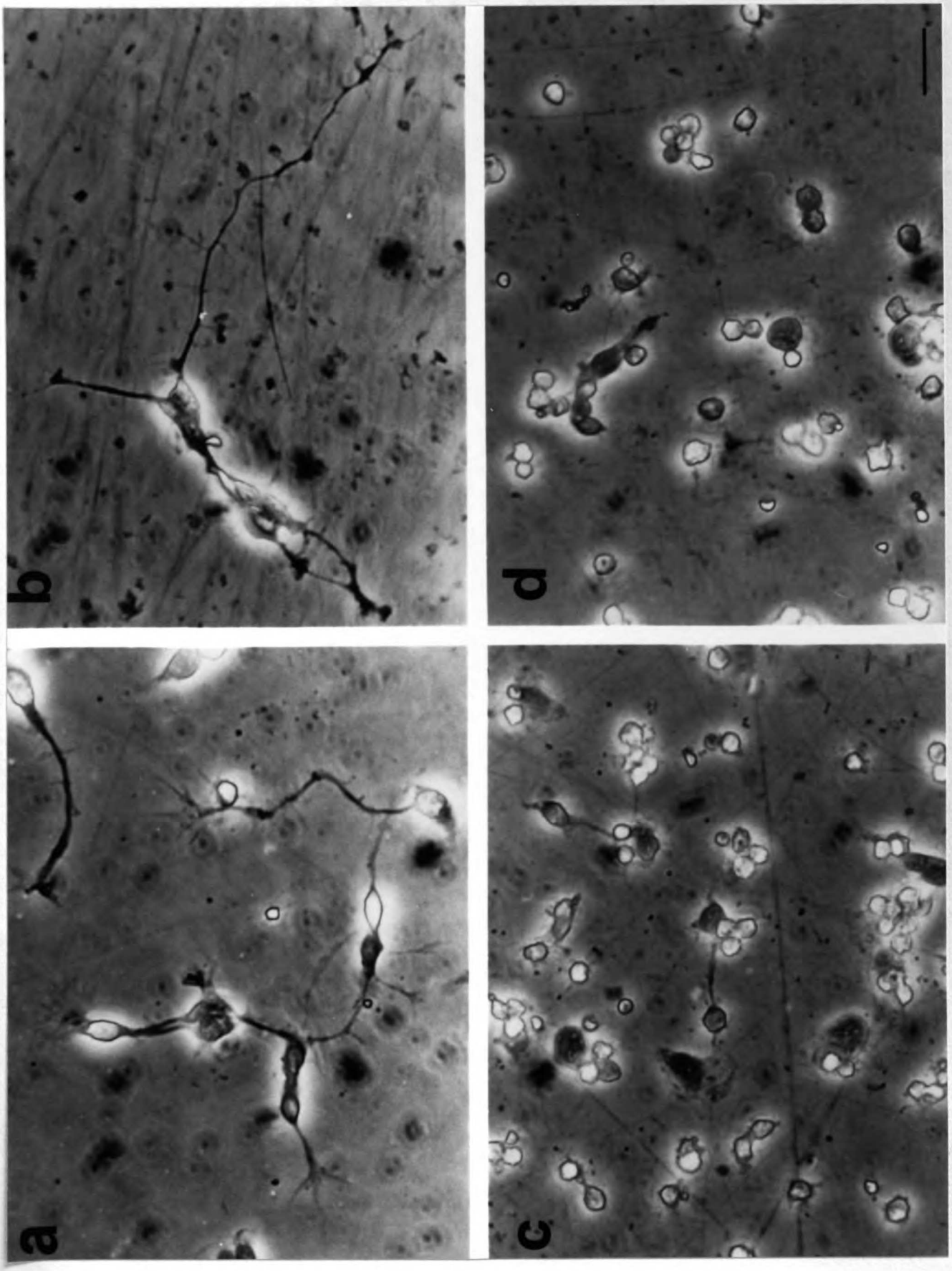
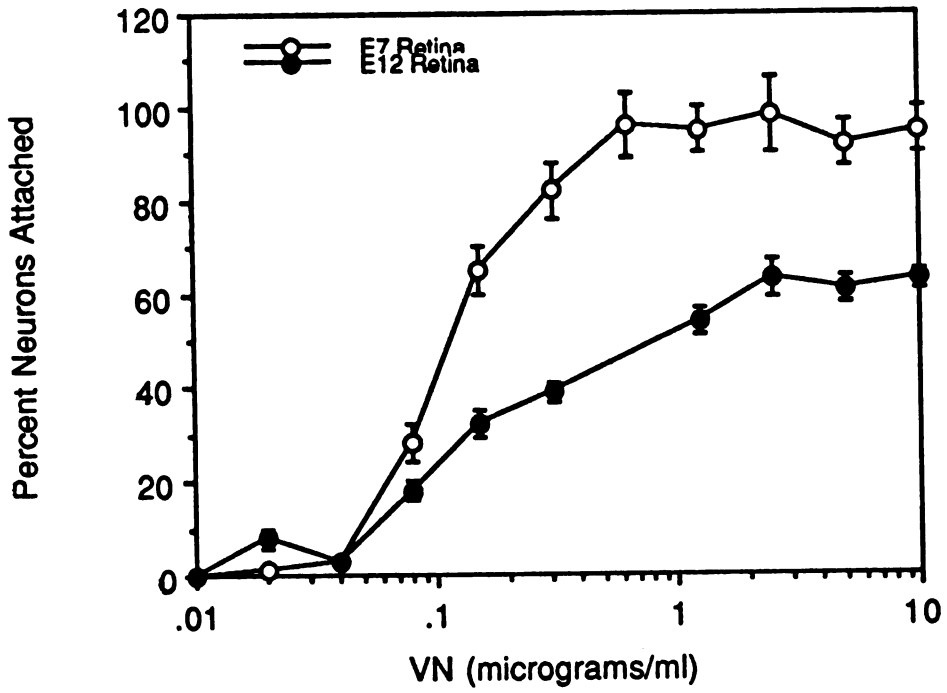

b
d
a
c

Figure 5.2: E7 and E11 retinal neuron attachment to VN and TSP. *a*) E7 retinal neuron attachment to VN after 1 hr was dose-dependent with 100% of the cells attaching to substrates coated with 0.8 µg/ml VN. ~60% E11 retinal neurons attached to VN with a similar dose-dependence. *b*) 80% of E7 retinal neurons attached to the highest concentrations of TSP tested (200 µg/ml) after 90 min. 40 % of E11 neurons attached to TSP. All values represent the mean +/- SEM of three determinations.


E7 Retina
E12 Retina
Percent Neurons Attached
120
100
80
60
40
20
0
.01
.1
1
10
VN (micrograms/ml)

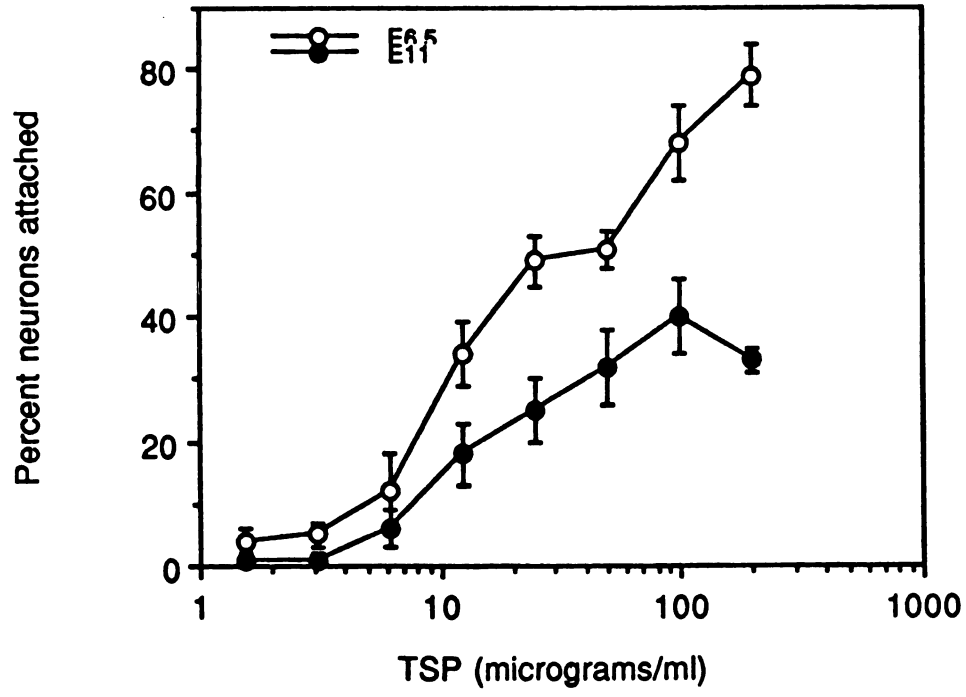

E6.5
E11
Percent neurons attached
80
60
40
20
0
1
10
100
1000
TSP (micrograms/ml)

Figure 5.3: Visualization of chicken VN by immunoblot using a polyclonal antibody. The purified immunogen migrates at 70kD is visible by Coomassie staining in lane 1. In immunoblots, a 70/74 kD doublet is detected in extracts of E7 (lane 2) and E11 (lane 3) retinae. A prominent 70kD band is detected in E7 lens (lane 4) and E7 vitreous humour (lane 5). Preimmune serum, lanes 6-9 in the same order.


1 2 3 4 5 6 7 8 9
97 —
66 —
43 —
31 —
Immune
Pre-immune

Figure 5.4: Immunolocalization of VN in the developing chicken optic nerve and retina. Bright field optics were used for photography, such that only the HRP reaction product and the pigment granules of the outer epithelium are visible. *a*) VN immunoreactivity was present in sections of E5 optic stalk and nerve. b) In the E6 retina, VN staining was detected surrounding cell bodies and, most strongly, in the inner limiting membrane adjacent to the optic fiber layer (arrowheads). c) Sections of E12 retina incubated with the preimmune staining demonstrate the lack of background staining, since only the pigment granules of the pigmented epithelium (PE) are visible. Scale bar pertains to b and d. d) In the E12 retina, the optic fiber layer and the inner (ipl) and outer (*) plexiform layers were strongly labeled. In addition, reaction product is visible surrounding cell bodies, in particular those of the ganglion cell layer (g). NR, neural retina; ON, optic nerve; PE (curved arrows), pigment epithelium. Scale bars: 20 υm.

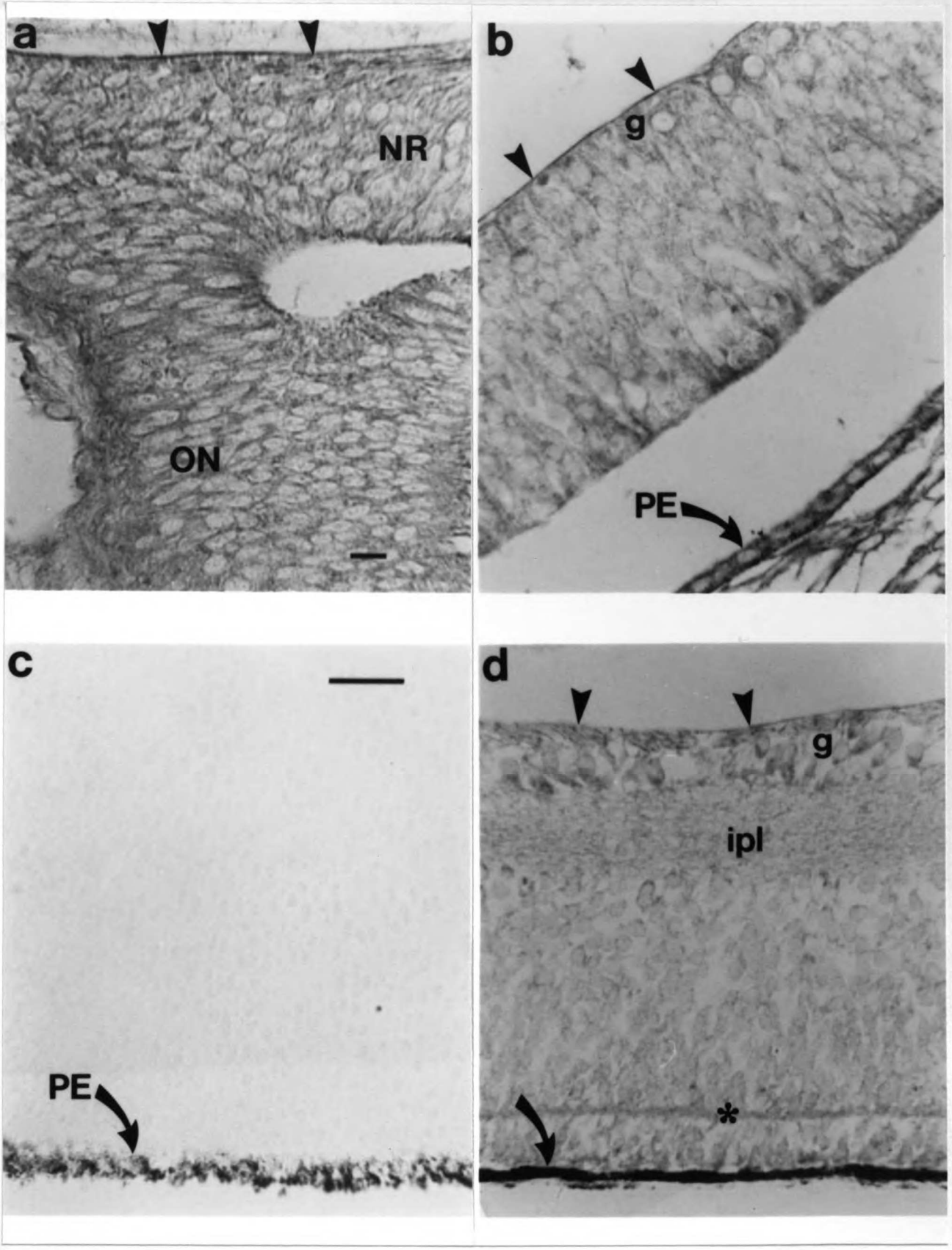

a
NR
ON
b
g
PE
c
PE
d
g
ipl
*

Figure 5.5: Identification of two monoclonal antibodies, Chav-1 and TASC, as inhibitors of E7 retinal neuron attachment to VN. In a time course experiment, maximal attachment to VN was acheived at 40 minutes in the absence of antibodies (filled circles). Both Chav-1 (filled squares) and TASC IgG's (open squares) decreased attachment most dramatically at early times, but continued to have effects at 1h. The effects of the antibodies were additive, since the combination of Chav-1 plus TASC (open circles) reduced attachment more effectively than either antibody alone. Both IgG's were used at 200 μg/ml.

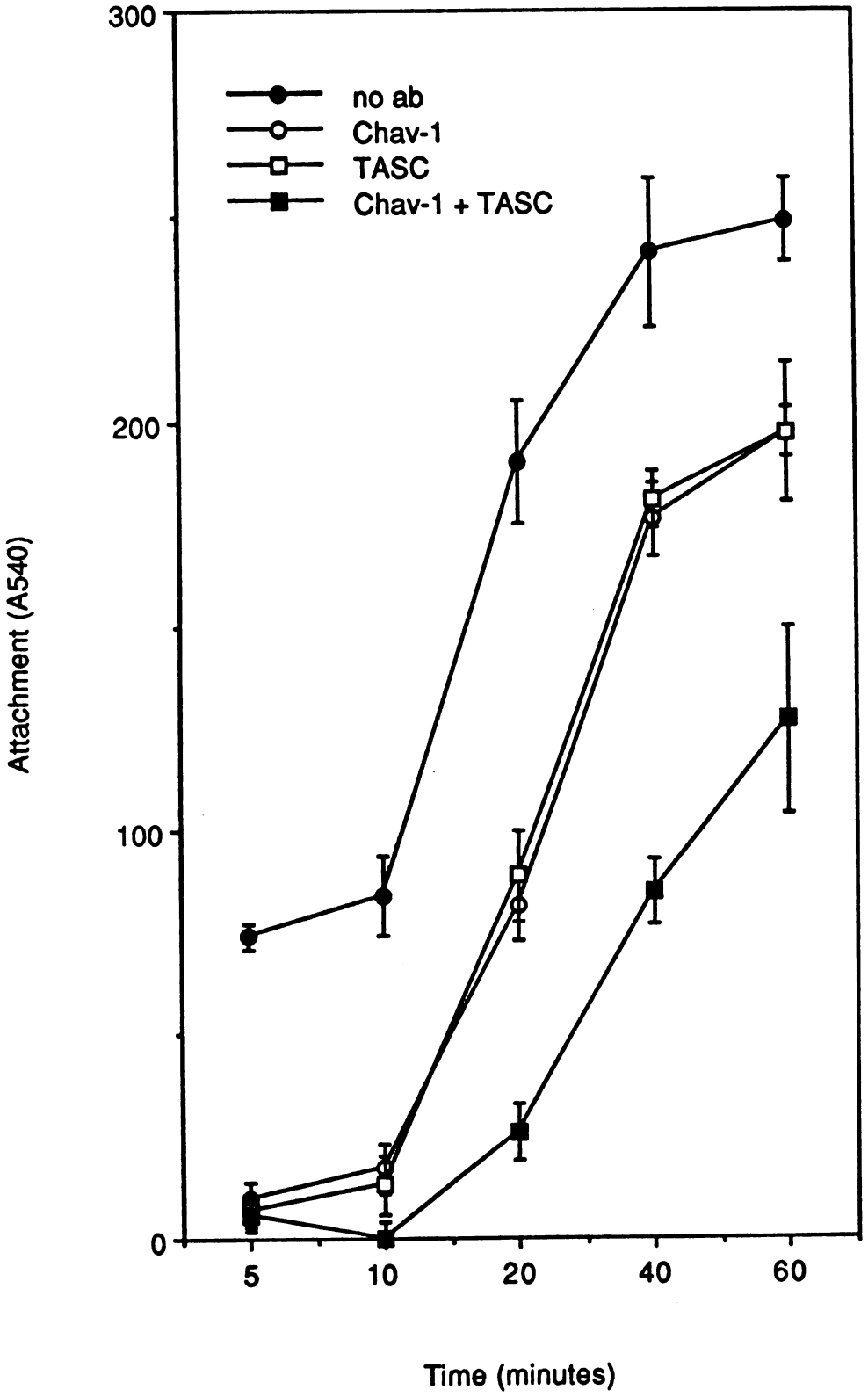
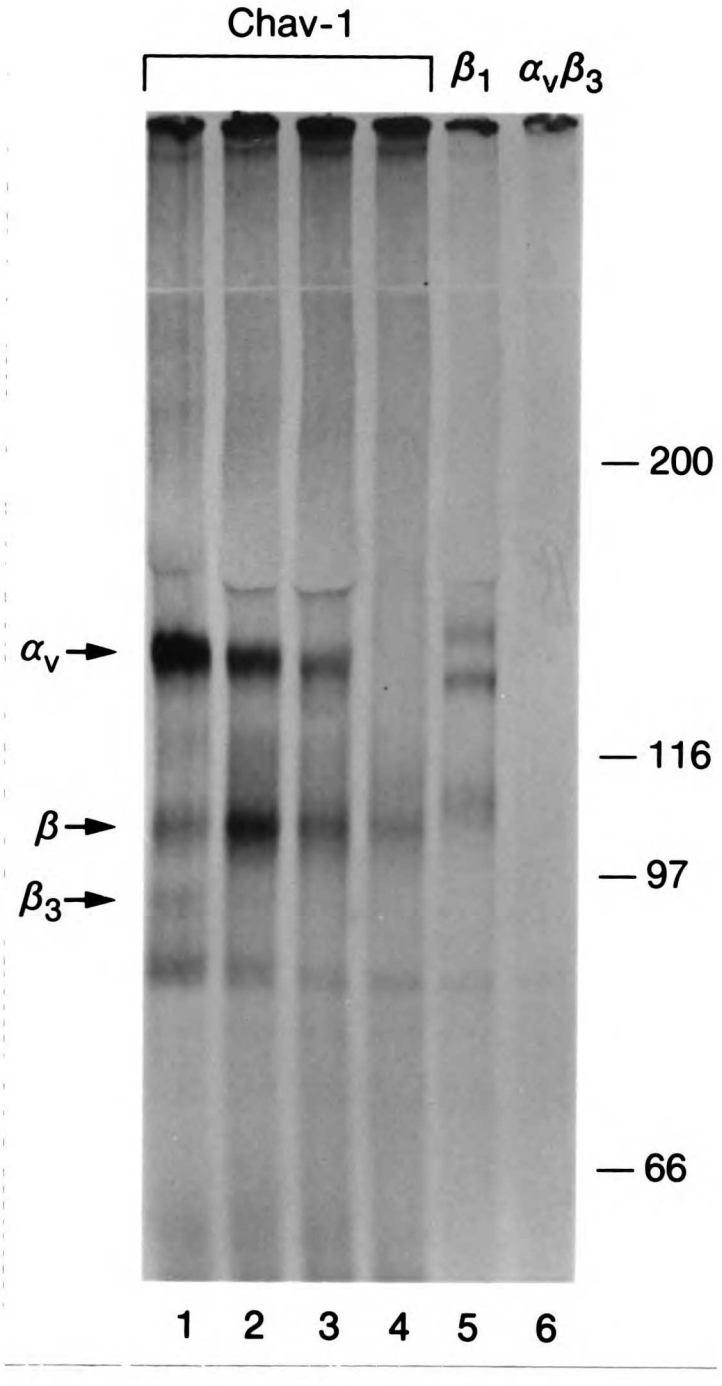
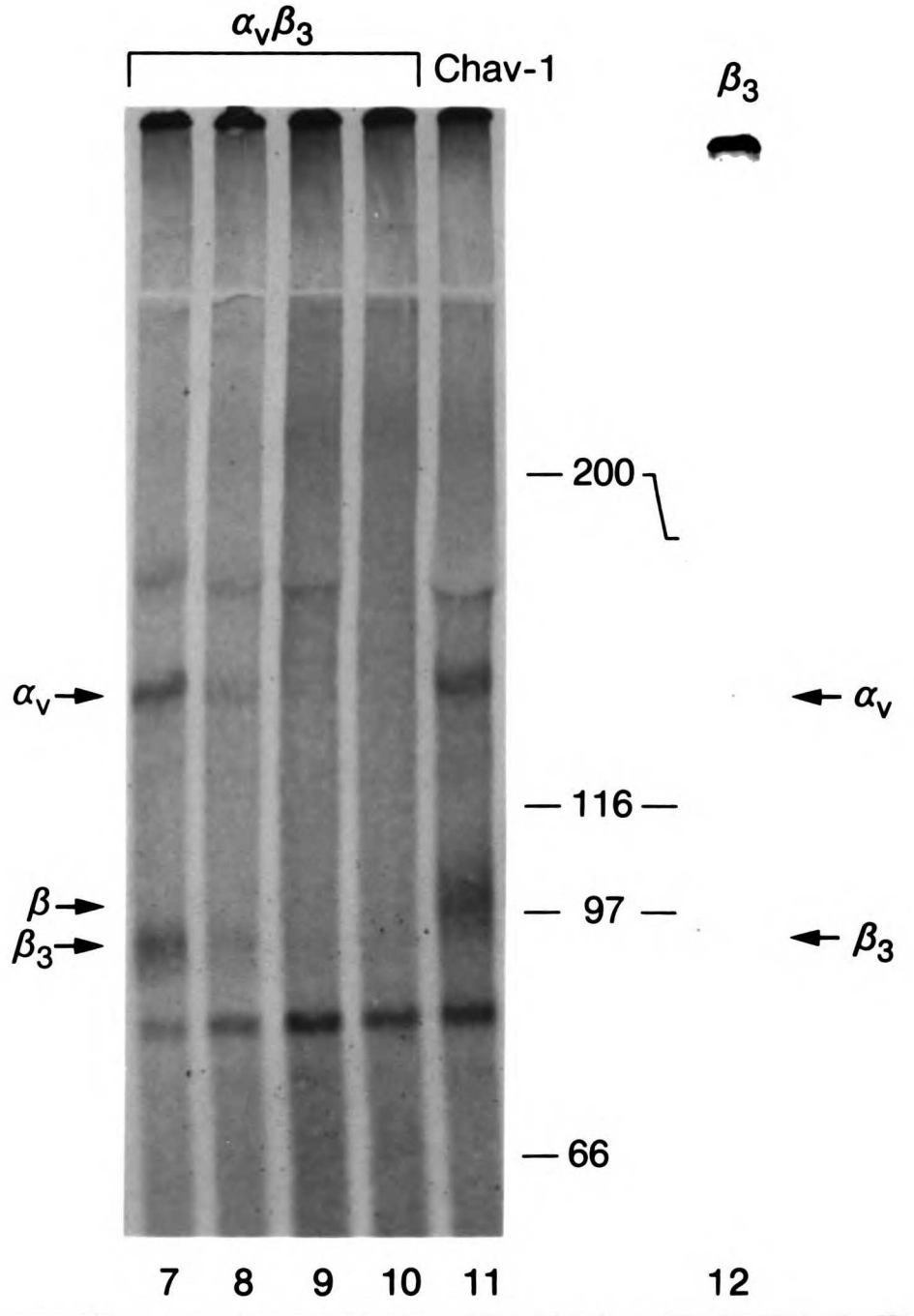

300
200
100
0
no ab
Chav-1
TASC
Chav-1 + TASC
Attachment (A540)
5
10
20
40
60
Time (minutes)

Figure 5.6: Characterization of the Chav-1 antigen. *a*) Immunoprecipitation of the Chav-1 antigen from ^{35}S-labeled E7 retinal neuron extracts in an immunodepletion experiment (lanes 1-4) reveals 3 specific bands at 140, 100, and 92kD resolved on 6% polyacrylamide gels under non-reducing conditions. Subsequent precipitation of the depleted extract with TASC (lane 5) resolves a panel of β_1-class integrins at 145, 135, and 105kD and shows that significant degradation of proteins in the extract has not occurred. LM609 fails to detect $\alpha_v\beta_3$ in the depleted extract (lane 6). Immunodepletion with LM609 (lanes 7-10) detects $\alpha_v\beta_3$ in the starting extract, but does not remove the 140 and 100kD material precipitated by Chav-1 (lane 11). A polyclonal antiserum to the cytoplasmic domain of the β_3 subunit precipitates only the 140kD and 92kD bands (lane 12). *b*) β_1 and α_v immunoblots of immunoprecipitated, reduced material from E7 retinal extracts. Polyclonal antibodies against the β_1 subunit detect their reduced antigen at 115kD in the TASC immunoprecipitate, but not in the Chav-1 or LM609 precipitates. Polyclonal antibodies against the heavy chain of the chicken α_v subunit detect their 130kD antigen in the Chav-1 immunoprecipitate, but not in the TASC or CSAT precipitates.

A.


Chav-1
β_1
$\alpha_v\beta_3$
— 200
— 116
— 97
— 66
α_v
β
β_3
1 2 3 4 5 6


αvβ3
Chav-1
β3
200
αv
αv
116
β
β3
97
β3
66
7
8
9
10
11
12

B.

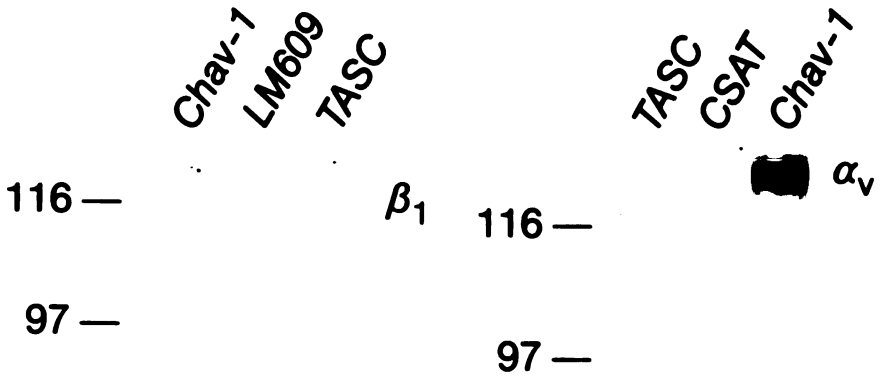
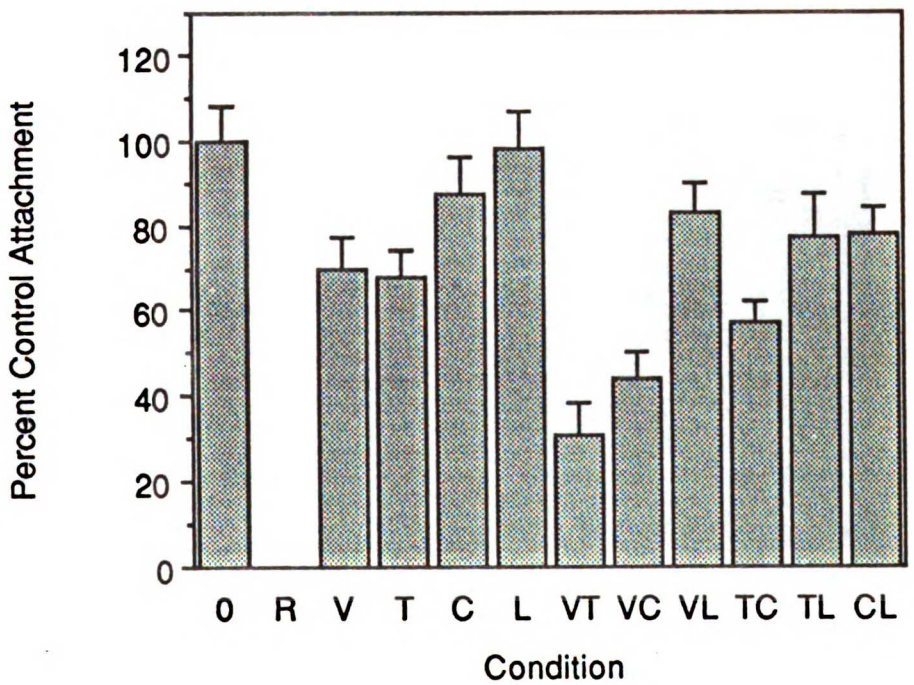
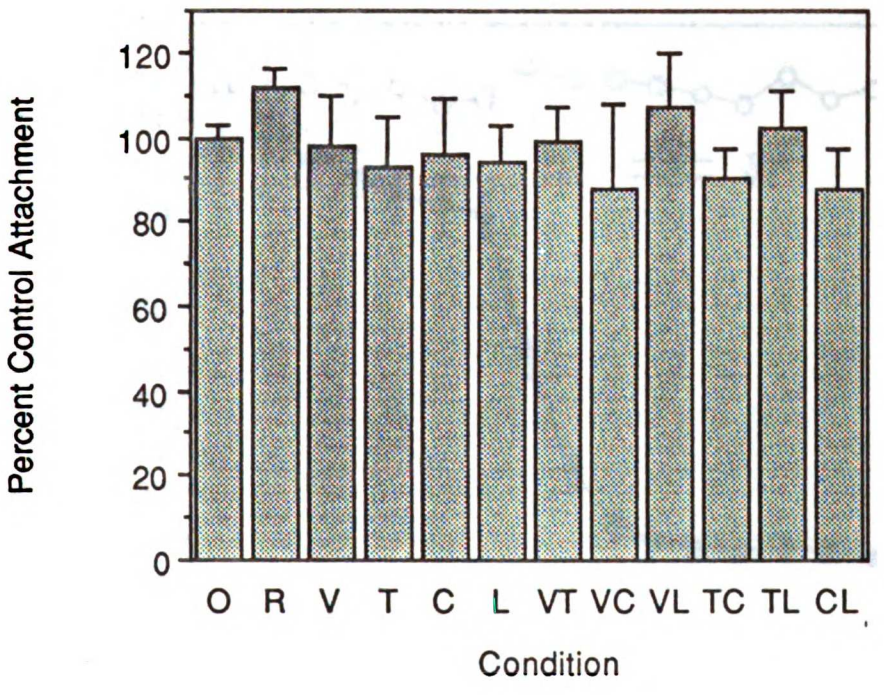


Figure 5.7: The effects of antibody combinations on E7 retinal neuron attachment to *a*) VN after 10 min of attachment and *b*) TSP after 90 min of attachment. Abbreviations are as follows: R, 100 μg/ml GRGDSP; C, 50 μg/ml CSAT; T, 200 μg/ml TASC; V, 100 μg/ml Chav-1; L, 100 μg/ml LM609. *c*) Soluble heparin blocks E7 retinal neuron attachment to TSP but not VN. Concentration curve of heparin added to the medium during 90 minutes of attachment.

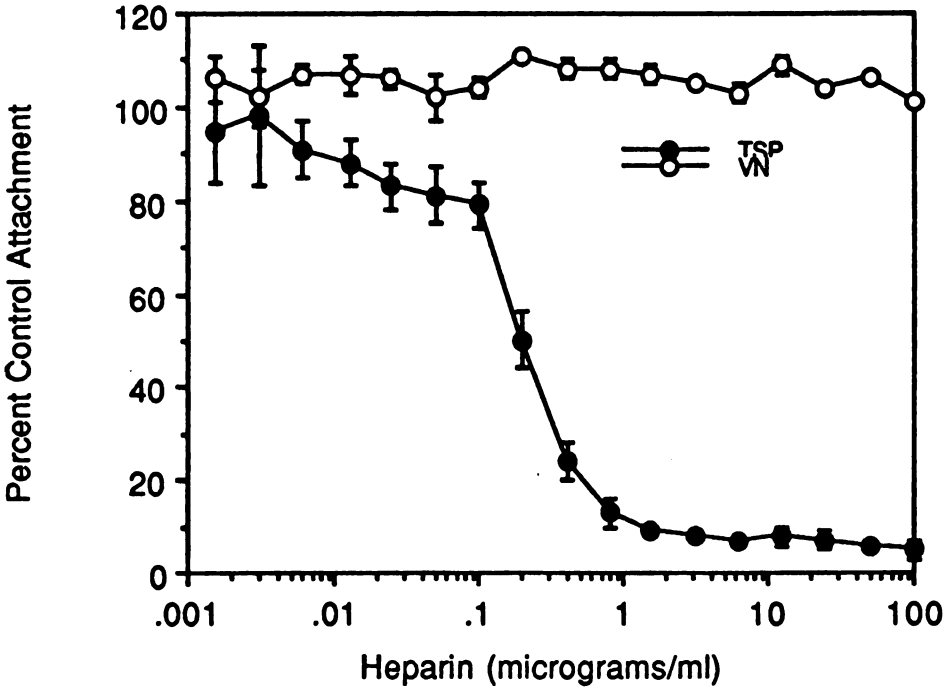
A.


Percent Control Attachment
120
100
80
60
40
20
0
0 R V T C L VT VC VL TC TL CL
Condition

B.


Percent Control Attachment
120
100
80
60
40
20
0
O R V T C L VT VC VL TC TL CL
Condition

C.


120
100
80
60
40
20
0
Percent Control Attachment
.001
.01
.1
1
10
100
Heparin (micrograms/ml)
TSP
VN

CHAPTER SIX

DISCUSSION

Developing neurons express cell surface receptors that mediate both cell adhesion and growth cone motility in response to specific protein ligands in vitro. The major focus of this work has been (1) to identify the receptors and their ligands that mediate adhesion and neurite outgrowth by embryonic chicken retinal neurons, and (2) to understand receptor activity in terms of the responses each transduces at various stages of development. This approach has led to one principal conclusion: that a receptor's ability to promote cell adhesion is separable from its ability to promote neurite outgrowth. Thus, although it is generally true that growth cones must adhere to a substrate to move on it, movement is likely to reflect specific receptor-mediated signals additional to those that underly the adhesion of the cell body to the ligand. Many of the receptors studied here are subject to modulation such that a given receptor may, at a given time and context during development, either perform a purely adhesive role or stimulate process growth in addition. Since such receptor-ligand pairs are expected to modulate cell interactions in developing neural tissue, this regulatory feature of receptor function provides a dynamic mechanism for determining neuronal position within the tissue and for specifying axonal and dendritic growth and guidance.

Two in vitro scenarios, one examining retinal neuron interactions with purified glycoprotein substrates and another with astrocyte surfaces, have facilitated studies of short term cell adhesion and the longer-term neurite outgrowth response. The neuronal receptors that promote adhesion and neurite outgrowth include members of the integrin family of ECM receptors and the cell adhesion molecules, N-cadherin, NCAM and L1. The activity of integrins completely accounts for retinal neuron responses to laminin, fibronectin, collagens types I and IV, and vitronectin. Integrins also promote neurite outgrowth on thrombospondin and astroglial cell surfaces. However, retinal neuron adhesion to thrombospondin is likely to be attributable to the additional activity of a heparan sulfate proteoglycan(s), and

responses to astrocytes are mediated, in large part, by the activity of the CAMs.

Several observations support the view that "adhesive" receptors transduce a distinct signal for neurite outgrowth. First, antibodies that have profound effects on neurite elongation on astrocyte monolayers (eg. anti-N-cadherin) do not reduce cell adhesion to the monolayer. Second, some receptors (eg. NCAM and integrins) are capable of mediating neuronal adhesion without stimulating neurite outgrowth. Thus, adhesion is necessary but not sufficient for neurite extension. Furthermore, the ability of particular receptors to promote adhesion and/or neurite outgrowth depends on the developmental age of the neuron in ways that to not appear to simply reflect the level of receptor expression. Together, these observations suggest that once a receptor is expressed at the cell surface, at least two potential signals can be transmitted across the plasma membrane, one mediating adhesion and a second eliciting motility. While results in chapter 4 suggest that the ability of β_1-class integrins to mediate adhesion is regulated by receptor conformation, the means by which adhesive receptors are coupled to a neurite outgrowth-promoting signal are mysterious. The latter is likely to involve receptor association with cytoskeletal elements and/or the regulation of second messenger systems.

The regulated function of integrins

Early (E6-7) embryonic retinal neurons attach and extend neurites on laminin, fibronectin, collagens, vitronectin and thrombospondin (see Table 6.1). β_1-class integrins are entirely responsible for both responses to the first three proteins (chapter 2) and the associated α subunits that comprise these heterodimers are currently being studied by others. $\alpha_1\beta_1$ is a known laminin and collagen receptor on rat and human neurons (Ignatius and Reichardt, 1988; K. Tomaselli, C. Emmett, and P. Doherty, unpublished observations). However, $\alpha_1\beta_1$ has been shown to bind the E1-4 proteolytic fragment of laminin to which retinal neurons do not respond (Hall

et al., 1990; D.E. Hall, unpublished observations), suggesting that $\alpha_1\beta_1$ is not responsible for retinal neurite outgrowth on laminin or collagen. Instead, it is likely that retinal neuron interactions with laminin reflect at least $\alpha_6\beta_1$ function, since they express this known laminin receptor in abundance and since mouse retinal neurite outgrowth on laminin is partially inhibited by a monoclonal antibody against α_6 (Hall et al., 1990; I. DeCurtis and L.F Reichardt, unpublished observations; J. Cohen, unpublished observations). However, other β_1-class laminin receptors -- $\alpha_2\beta_1$, $\alpha_3\beta_1$,and $\alpha_7\beta_1$ -- have been identified in other systems and may also play a role (cf. Takada, et al., 1988; Kramer et al, 1989).

The integrin $\alpha_5\beta_1$ heterodimer is a major arg-gly-asp-sensitive receptor for fibronectin and is expressed by dorsal root ganglion neurons which grow extensive neurites on fibronectin (Pytela et al., 1985; C. Weaver, unpublished results). However, results presented in Appendix A suggest that E7 retinal neurons interact with fibronectin predominantly through the CS1 domain of fibronectin rather than through the arg-gly-asp-containing cell binding domain. $\alpha_4\beta_1$ has been shown to bind this domain of FN and is therefore a possible mediator of retinal neuron adhesion to CS1 (Wayner et al., 1989; Elices et al., 1990; Mould et al., 1990). The finding that retinal neurons of any age respond to fibronectin is surprising since FN expression is not detectable in the developing chick retina (Halfter and Fua, 1987). Certainly, the levels of FN may be too low or the antigenic determinants inaccessible to permit the detection of FN. On the other hand, one may speculate that attachment and neurite outgrowth in response to FN reflects the ligand-specificity of the receptor $\alpha_4\beta_1$ which also binds VCAM, an endothelial cell adhesion molecule of the immunoglobulin superfamily (Elices et al., 1990). It is interesting to consider that VCAM or a similar CAM may be expressed in developing nervous tissues where it might support adhesion and/or neurite outgrowth via $\alpha_4\beta_1$. VCAM expression on the surfaces of other axons, Muller glia,

or astroglial precursors in the optic nerve would be strongly suggestive of such a function. Unfortunately, localization of VCAM in the embryo has not been reported, leaving this question open to investigation.

In contrast to retinal neuron interactions with laminin, collagens and fibronectin, β_1-class integrins have only partial roles in responses to vitronectin and thrombospondin. Attachment to vitronectin was partially inhibited by a β_1-specific monoclonal antibody (TASC), and a non-β_1-class α_v/100kDβ integrin heterodimer was also implicated (chapters 4 and 5). Attachment to thrombospondin was not dependent on the activity of α_v- and β_1-containing integrin heterodimers and instead appeared to reflect interactions with the heparin-binding domain of thrombospondin, perhaps by a heparan sulfate proteoglycan(s). Nevertheless, β_1-class integrins played the dominant role in neurite outgrowth on thrombospondin, since a β_1-specific monoclonal antibody (CSAT) nearly abolished this response (chapter 5). Interestingly, retinal neurons express another class of integrin heterodimer, $\alpha_v\beta_3$, which has been shown to bind both vitronectin and thrombospondin (Cheresh and Spiro, 1987; Lawler et al., 1988). However, an $\alpha_v\beta_3$ function-blocking monoclonal antibody (LM609) that inhibits $\alpha_v\beta_3$ function on chicken myeloblasts did not reduce neuronal attachment or neurite outgrowth in response to either ligand (chapter 5; appendix B). Thus, retinal neurons appear to express an integrin heterodimer that has no obvious function, illustrating the point nicely that the mere expression of any protein by a cell does not demonstrate function.

β_1-class integrins also mediated retinal neurite outgrowth on astrocyte monolayers. Astrocytes are known to synthesize laminin, fibronectin, and thrombospondin (Liesi et al., 1983; Price and Hynes, 1985; Asch et al., 1986). Collagen and vitronectin synthesis by astrocytes has not been examined. However, astrocytes are cultured in 10% fetal calf serum which contains large amounts of

soluble vitronectin. Thus, regardless of astrocyte expression of this protein, serum vitronectin may have been adsorbed onto the astrocyte surfaces. Since the activity of β_1-class integrins is important in neurite outgrowth on all of these purified proteins, each is a potential ligand on cultured astrocytes.

By a later stage in development (E11), retinal neurons no longer adhere to purified laminin or fibronectin, and fewer adhere to vitronectin and thrombospondin. While they remain able to attach to collagen IV, vitronectin and thrombospondin, they do not extend neurites on any of these molecules. It is likely that β_1-class integrin function is regulated post-translationally on these neurons for several reasons: (i) the profile of β_1-class integrins on E11 neurons does not reveal a loss of α subunits expressed by the E6 retinal neuronal population. In fact, an additional α band appears at E11 (chapter 2). (ii) β_1-class integrins continue to function in E11 retinal neurite outgrowth on astrocytes (chapter 3). (iii) Integrins are expressed at sufficiently high levels on E11 retinal neurons to support at least partial attachment to collagen I and IV, and vitronectin (chapters 2 and 5). (iv) E11 retinal neuron attachment to laminin can be induced by the TASC monoclonal antibody that was shown to increase β_1-class integrin function (chapter 4). These points argue that the activity of integrin β_1-class heterodimers in promoting neurite outgrowth is regulated independently of their attachment-promoting ability. In addition, the last observation suggests that laminin receptor function in both events is subject to modulation. It is also noteworthy that α_v/100kDβ function must also be developmentally regulated, since this heterodimer produces E11 neuron attachment to vitronectin but not neurite outgrowth. Considerable evidence exists for the dynamic regulation of integrin function on immune cells and platelets where conformational changes, cytoskeletal association, and the action of second messenger systems have been implicated (Plow and Ginsberg, 1989; Shaw et al., 1990; Shimizu et al., 1990; Springer, 1990). That these phenomena should be

implicated in integrin function on developing neurons is exciting and challenging.

Potential loci for integrin functional modulation include the regulation of integrin receptor phosphorylation which has been demonstrated on at least β_1, β_5 and α_6 (Hirst et al., 1986; Freed et al., 1989; Shaw et al., 1990) and/or specific association with cytoskeletal elements (Burn et al., 1986; Shaw et al., 1990), both of which are likely targets of second messenger activity. Phosphorylation of α_6 is associated with receptor activation, whereas phosphorylation of chicken β_1 and an α subunit component is correlated with decreased function (Hirst et al., 1986; Shaw et al., 1990). Interestingly, ablation of retinal ganglion cell target tissue, the optic tectum, reduces the loss of retinal ganglion cell response to laminin (Cohen et al., 1989). Similarly, regulated laminin receptor function on ciliary ganglion neurons appears to correlate with their innervation of peripheral targets (Tomaselli and Reichardt, 1988). These observations suggest that neuronal contact with target cells or with molecules, such as neuronotrophic factors, secreted by the target alters integrin expression and/or function. Indeed, α_6 mRNA levels in retinal ganglion cells decrease between E6 and E11, but a similar decrease is not detected in non-ganglion cell retinal neurons (I. DeCurtis, unpublished observations). Retinal ganglion cells respond to brain-derived neurotrophic factor (BDNF) which interacts with the low affinity NGF receptor, and NGF receptor mRNA is abundant in the chick neural retina (Johnson et al., 1986; Large et al., 1989; Rodriguez-Tebar et al., 1990). Treatment of PC12 cells with NGF results in the phosphorylation of the actin-associated protein, vinculin, and its translocation to the growth cone (Halegoua, 1987). Thus, there is precedence for growth factor-mediated alterations in the phosphorylation state of growth cone cytoskeletal elements, indicating that phosphorylation by specific kinases may regulate the ability of integrins to transduce the neurite outgrowth response.

Expression of integrin ligands in the developing neural retina

Laminin, collagen IV, and thrombospondin immunoreactivity have been described in the developing neural retina (Cohen et al., 1987; Halfter and Fua, 1987; O'Shea and Dixit, 1988). The distribution of laminin undergoes developmental changes such that in the early embryo, laminin is present in the extracellular spaces of the neuroepithelium, in the basement membrane of the optic fiber layer (the inner limiting membrane), and in the optic stalk. By E8, however, laminin disappears from the stalk and cellular layer of the retina and is instead confined to the inner limiting membrane (Cohen et al., 1987; Halfter and Fua, 1987). This decrease in laminin expression is, therefore, correlated with the loss of laminin receptor function by retinal neurons as a whole.

Vitronectin was localized to the developing neural retina (chapter 5) where is was found in the extracellular spaces of the E7 neural epithelium and ganglion cell layer, as well as the inner limiting membrane. Consistent with its continued function as an adhesion molecule at later stages in development, vitronectin was present in the plexiform layers, surrounding the cell bodies of ganglion cells and in the other nuclear layers, and in the inner limiting membrane at E12. Vitronectin was also prominently expressed in the developing brain and peripheral nervous system of the chick (C.J. Emmett and K.M. Neugebauer, unpublished observations), suggesting that vitronectin also plays an important role in the development of the nervous system outside of the retina.

The regulated function of CAMs

Both the Ca^{++}-dependent CAM, N-cadherin, and the Ca^{++}-independent CAM, NCAM, were shown to promote neurite outgrowth by retinal neurons on astrocyte surfaces (chapter 3). Subsequently, N-cadherin and an additional CAM, L1, have been shown to promote retinal ganglion cell neurite outgrowth on Muller

glial cells (Drazba and Lemmon, 1990). All three molecules are present in the retina and in the optic nerve, consistent with their potential function in vivo (Lemmon and McLoon, 1986; Matsunaga et al., 1988b; Bartsch et al., 1989). N-cadherin is expressed in the optic fiber layer and in the neuroepithelium at early times in development, suggesting that N-cadherin on the surfaces of other axons as well as on other cells regulates process growth in the eye and optic nerve. NCAM has been shown to play a role in the ordered growth of axons in the optic nerve and in the specification of axon interactions with tectal targets (Fraser et al. 1984; 1988; Silver and Rutishauser, 1984; Thanos and Bonhoeffer, 1984). L1 expression in the retina is confined to the optic fiber layer, suggesting that it can function in L1-L1 interactions within fascicles and in optic fiber interactions with cells expressing a heterologous ligand (Grumet and Edelman, 1988; Drazba and Lemmon, 1990).

N-cadherin played a major role in astrocyte-stimulated neurite outgrowth by both E7 and E11 retinal neurons. However, NCAM had no detectable function in E7 retinal neurite outgrowth on astrocytes, even though it contributed significantly to adhesion. In contrast, antibodies to NCAM dramatically reduced E11 retinal neurite outgrowth on astrocytes. Thus, like integrin receptors for collagens and vitronectin, NCAM can play a purely adhesive role even though it is capable of promoting neurite outgrowth in another developmental context. One possibility is that E11 retinal neurons express slightly higher levels of a relevant molecular form of NCAM, since a threshhold level of NCAM has been shown to required for it to promote neurite outgrowth (Doherty et al., 1990a). No change in NCAM levels was detected on the growth cones of retinal ganglion cells from E6 to E11 (Doherty et al., 1990b), however these authors found that NCAM was more effective in promoting ganglion cell outgrowth at E6 than at E11. Alternatively NCAM function could be subject to regulation by cytoskeletal connectivity as discussed for the integrins above. Interestingly, astrocyte maturation is associated with a loss of

neurite outgrowth-promoting activity which is likely to be an important factor in the inability of CNS neurons to regenerate (Smith et al., 1986). A recent report shows that this change is due to the loss of NCAM and L1-dependent mechanisms (Smith et al., 1990). In vivo, therefore, the regulation of CAM activity on both neuronal and astrocyte surfaces is likely to determine their function.

Relevance to the specification of the optic projection

This work has documented the activity of specific integrins and CAMs in promoting retinal neuron adhesion and neurite outgrowth in response to molecules present in the optic pathway. Although the entire retinal neuron population was studied, and retinal ganglion cells were not singled out, some speculation on the potential roles of these molecules in the optic pathway can be entertained. Certainly, studies of retinal ganglion cell outgrowth support the view that integrins, N-cadherin, and NCAM are active on these cells (Cohen et al, 1987; Doherty et al., 1990b; Drazba and Lemmon, 1990). Furthermore, the functions of laminin, fibronectin, vitronectin, and thrombospondin receptors on ganglion cells are likely to undergo developmental regulation, since the entire retinal neuron population exhibited such changes in these studies and laminin receptor regulation on retinal ganglion cells has been directly shown (Cohen et al., 1987; DeCurtis et al., submitted).

Of particular interest are the mechanisms underlying the specification of the retino-tectal map. Retinal ganglion cells project with topographic specificity to their targets in the tectum, providing a popular model system for the specification of neural connections in general. Sperry's chemoaffinity hypthesis (1963) was the first attempt to explain, in molecular terms, how this specificity arises. He predicted that each afferent axon and each tectal partner bore chemical labels that dictated final connectivity. It was subsequently appreciated that (1) when a portion of the retina is

removed, the remaining retinal ganglion neurons distribute over the entire surface of the tectum such that they synapse with tectal neurons that would not normally be their targets (reviewed in Fraser and Hunt, 1980), and (2) as the tectum grows and as new fibers reach the tectum, pre-existing afferent connections actually "slide" across the tectum (cf. Gaze et al., 1979; Fraser, 1983; McLoon, 1985). Therefore, a strict lock-and-key mechanism for ganglion cell connections with tectal targets is unlikely. Instead, the chemoaffinity hypothesis has been modified to include a number of factors, such as putative gradients of molecules, competetive interactions between axon terminals, and synaptic rearrangement due to electrical activity (cf. von der Malsburg and Willshaw, 1977; Fraser, 1980, 1985; Schmidt, 1982; Gierer, 1983; 1987).

Fraser's model (1980; 1985) for the development of the retino-tectal projection will be considered in most detail, since it makes important predictions about the nature of the chemical signals that impart specificity. In this model four classes of interactions are arranged in a hierarchical order. First and dominant, should be a biochemically based attraction, called "C", between afferents and the tectal surface. In simulations of the model, "C" works best if it has homophilic binding properties. Second in importance is a repulsive interaction, "R", between nerve terminals which would drive arriving afferents to a uniform distribution over the tectum. "DV", a weak position-dependent adhesion between afferents and the tectum over the dorsal-ventral axis of the target, and "AP", an even weaker position-dependent cue in the perpendicular tectal axis, are both predicted to be mediated by a homophilic mechanism. Note that homophilic adhesion molecules are invoked for C, DV, and AP because they are the most informative, serving to maintain neighbor-neighbor interactions while also specifying positions on the tectum.

Since the elaboration of this model, some progress has been made in obtaining potential C, R, DV, and AP molecules or at least evidence that supports

their existence. For example, posterior but not anterior tectal membranes have been shown to contain a phosphatidylinositol-linked protein that repels temporal afferents, consistent with the topology of the projection (cf. Walter et al, 1990). Further, two antigens have been shown to have a polarized distributions: the TOP antigen which is most abundant in the dorsal retina and ventral tectum and which has been implicated in synapse formation within the retina (Trisler et al., 1986; Trisler and Collins, 1987), and the JONES antigen, a ganglioside prominent in the dorsal retina (Constantine-Paton et al., 1986). However, the functional capabilities of TOP and JONES in the development of the retino-tectal projection are unknown.

Studies on the role of NCAM support its function as a likely C molecule. NCAM antibodies disrupt the order of axonal growth on the optic nerve (Silver and Rutishauser, 1984; Thanos et al., 1984). When delivered to the tectum, NCAM antibodies produce an expansion of receptive fields across the whole of the tectum (Fraser et al., 1984; 1988). In the chick, the first retinal ganglion cell axons reach the tectum at E6 (cf. Rager, 1980), suggesting that their growth cones have the activity of integrins, N-cadherin, and L1 in addition to NCAM at their disposal. L1 and N-cadherin function has not been examined in this system, but might also represent C molecules. Interestingly, L1 in the tectum is exclusively localized to optic fibers (Lemmon and McLoon, 1986), suggesting that it might act in a homophilic fashion to mediate axon-axon interactions and in a heterophilic fashion to mediate afferent interactions with the tectum.

Brief contemplation of the potential roles for these cell adhesion molecules in optic pathway development, brings the contemplator to a paradox. *How can the same molecules that promote growth cone motility and the directed growth of axons also specify points in the pathway where the axons should stop?* The activities of several inhibitors of growth cone motility have been described, raising the possibility that an inhibitor at the target might suffice to tell the growth cone to stop

growing. However, these inhibitors have been shown to cause growth cone collapse and retraction even on permissive substrates (cf. Cox et al., 1990; Davies et al., 1990; Raper and Kapfhammer, 1990). This would seem to be an inappropriate response to a potential tectal partner, since collapse and retraction are unlikely to culminate in synapse formation. Nevertheless, these extreme responses to inhibitors may be a function of the in vitro systems used. Thus, the action of inhibitors at the target remains a formal possibility.

Another explanation is that the nature of the signals tranduced by these molecules is transformed from one that promotes growth cone motility to one that promotes adhesion and synaptogenesis. We already know that the neurite outgrowth-promoting ability of at least integrins and NCAM is separable from their ability to promote cell adhesion as discussed above. How might the switch in activity be achieved at the tectum? A number of possibilities exist: (1) As in examples of regulated laminin receptor function on retinal ganglion cells and ciliary ganglion neurons, factors supplied by the target might modify the activity of other receptors (Tomaselli and Reichardt, 1988; Cohen et al., 1989). (2) Molecular variants of some of these molecules may transmit the signal to stop rather than go. Precedence for this is provided by the neuromuscular synapse-specific form of laminin, S-laminin, which may help target regenerating motorneuron growth cones to previous junction sites (Hunter et al., 1989a & b). (3) The aquisition of afferent electrical activity might suppress growth cone motility -- as suggested by the work of Kater and colleagues (1988) -- while preserving adhesion. (4) Changes in receptor activity may be dictated by the developing neuron which itself shuts off the machinery required for the neurite outgrowth response. For example, the progressive loss of integrin-dependent neurite outgrowth by retinal neurons correlates temporally with the innervation of the tectum (E9-E12; Rager, 1980) and could reflect the downregulated expression of, say, a particular actin-associated

protein that links the integrin to motility. (5) Just as there is a set point for permissive levels of intracellular calcium ions (Kater et al., 1988), there may be an analogous set point for adhesion. Perhaps, the set of adhesive signals at the tectum combine to transduce adhesion in preference to motility by somehow altering growth cone cytoskeletal dynamics. The differential adhesion hypothesis predicts that during histogenesis, cells sort into layers with the highest affinity interactions internal and the lowest external (Steinberg, 1970). Since the neuronal cell body is relatively stationary during axon outgrowth, the growth cone might be imagined as a less adhesive "cell", wandering through tissue to the point where it attains a level of adhesiveness with which it is in equilibrium. In other words, the energy required for the growth cone to move away from its stopping place must exceed that of adhesion.

While each of these possibilities seems reasonable, there is little experimental evidence to endorse any real mechanism underlying the growth cone's capacity to recognize and cease growth at the target. It is likely that several factors will be important. As in pathfinding, the molecular choices available to the growth cone at any given point will determine its decision to move or not to move. A combination of molecules is likely to be important, since the neutralization of any one molecule at the tectum (eg. NCAM) does not destroy the growth cone's capacity to stop and form synapses. Finally, the context of the molecular recognition event will determine the signals transduced, since growth cone motility can be influenced by intrinsic factors such as internal calcium concentration. Among future prospects, it is imperative that we begin to understand the molecular nature of the signals transmitted to cells and growth cones during the processes of both adhesion and motility.

Table 6.1 Molecules that regulate retinal neuron adhesion and neurite outgrowth.

ligand	neuronal receptor	E6-7	E11
N-cadherin	N-cadherin	A,N	A,N
NCAM	NCAM	A	A,N
L1, ?	L1	N	nd
?	F11	F	nd
?	Neurofascin	F	nd
Laminin	β_1-class ($\alpha_6\beta_1$)	A,N	A*
Fibronectin	β_1-class	A,N	-
Collagens I and IV	β_1-class	A,N	A
Vitronectin	$\alpha_v\beta_?$, $\alpha_?\beta_1$	A,N	A
Thrombospondin	HeSPG(s) $\alpha_?\beta_1$	A,N	A

Key: A: attachment

A*: attachment (-) but inducible by TASC

N: neurite outgrowth

F: fasciculation

nd: not done

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APPENDIX A

The CS1 Domain of Fibronectin Promotes Attachment of Retinal Neurons

Embryonic chick retinal neurons interact with fibronectin (FN) in a developmentally regulated fashion. Specifically, E6 and E7 retinal neurons attach and extend neurites on FN while neurons from embryos older than E7 neither attach nor extend neurites on the same substrate (chapter 2). The structure of FN (shown in figure A.1) can be described in terms of domains of repeated homology, cell-binding, and binding of extracellular molecules. This modular structure allows for the assembly of FN into extracellular matrices and basal laminae by virtue of binding to collagen and heparin (Ruoslahti, 1988). In addition, several disinct domains are involved in cell binding, and these have been traditionally separated by proteolytic digestion. The "cell attachment" site is contained in a 120kD chymotryptic fragment; it bears the Arg-Gly-Asp sequence known to be important in cell interactions with FN as well as a "synergistic site" that enhances the activity of the cell attachment site (Obara, et al., 1988).

Another site, called CS1, is found C-terminal to the cell attachment site and consists of 25 amino acids that are encoded by an exon fragment that is alternately spliced into FN transcripts at a frequency determined by cell type (Paul et al., 1986). A synthetic peptide containing the CS1 sequence and conjugated to IgG promotes neurite outgrowth by dorsal root ganglion neurons in a fashion additive with a fragment containing the cell attachment site (Humphries et al., 1988). In another example, neural crest cells attach to either site alone, but spread and migrate only on the combination of CS1-IgG plus the cell attachment site (Dufour et al., 1988). Both of these studies suggest that neural crest cells and dorsal root ganglion neurons express receptors for the RGD-dependent cell attachment site and for CS1 which does not contain the RGD sequence.

As predicted from the cell biology, distinct receptors recognize the two cell-binding sites of FN. The integrin heterodimer $\alpha_5\beta_1$ binds FN in an RGD-dependent manner and is thought to be responsible for cell interactions with the cell attachment

site. The integrin $\alpha_4\beta_1$ heterodimer binds the CS1 domain of FN (Wayner et al., 1989). Interestingly, $\alpha_4\beta_1$ on the surface of lymphocytes also binds VCAM, an adhesion molecule on activated vascular endothelium that likely directs leukocytes to sites of inflammation (Elices, et al., 1990). An additional integrin, $\alpha_3\beta_1$, also binds FN, but the site it binds is currently unknown (Wayner and Carter, 1987; Takada et al., 1988).

In an effort to identify the FN receptor expressed by E6-7 retinal neurons, an antibody against a peptide from the cytoplasmic domain of α_5 was used in immunoprecipitation experiments. Little or no α_5 was detected in labeled E6 retinal neuron extracts, suggesting that $\alpha_5\beta_1$ is not the FN receptor expressed by retinal neurons. Since antibodies against α_4 and α_3 were not available, another approach was pursued. CS1 and the 120kD fragment of FN (FN120) were tested for their abilities to support attachment of E7 retinal neurons. While FN120 did not support attachment, CS1 did so in a β_1-dependent fashion. This suggests that $\alpha_4\beta_1$ or perhaps $\alpha_3\beta_1$ are the FN receptors expressed by developing retinal neurons. The observation that the addition of TASC, a monoclonal antibody that promotes integrin β_1 function, results in significant but low levels of attachment to FN120 is consistent with the possibility that retinal neurons express very low levels of integrins that bind the cell attachment site of FN.

RESULTS

The CS1 peptide (DELPQLVTLPHPNLHGPEILDVPST-C) was synthesized and conjugated via its C-terminal cysteine to keyhole lympet hemocyanin (KLH). Figure A.2 shows that CS1-KLH substrates promoted the attachment of 50% of E7 retinal neurons. The same level of attachment was observed previously on whole FN (chapter 2). No attachment was observed on KLH alone. Attachment to CS1-KLH was blocked by CSAT, a monoclonal antibody that inhibits the function of β_1-class integrins, but not by an RGD-containing hexapeptide. Thus, CS1 can account for all of the attachment-promoting activity of whole FN that was found to be integrin β_1-dependent (chapter 2).

To determine whether retinal neurons also respond to the cell attachment site of FN, FN120 was assayed for its ability to promote attachment. Only 7% of E7 retinal neurons attached to FN120 (fig. A.3). The monoclonal antibody TASC (described in chapter 4) has been useful in implicating the presence of β_1-class integrins whose functions are not detectable in some assays. The addition of TASC IgG promoted the attachment of E7 retinal neurons to FN120 but only to a low level, 22% (fig. A.3). This is consistent with the finding that little or no $\alpha_5\beta_1$ is detectable upon immunoprecipitation from labeled E6 retinal neurons (data not shown).

Figure A.1: Schematic representation of the localization of different cell binding sites (synergistic adhesion site, RGDS site, CS1 in the IIICS domain) along the fibronectin molecule and their possible role in cell attachment, spreading and migration of neural crest cells. One of the two chains comprising FN is represented. The other major binding domains (ie. collagen, fibrin I and II, and heparin I and II) are also indicated. The "motility domain" is shown covering the various cell-binding sites and other regions of the molecules, because it cannot be excluded that areas of the molecule distinct from the cell-binding domains can contribute to the motility-promoting activity. (From Dufour et al., 1988)

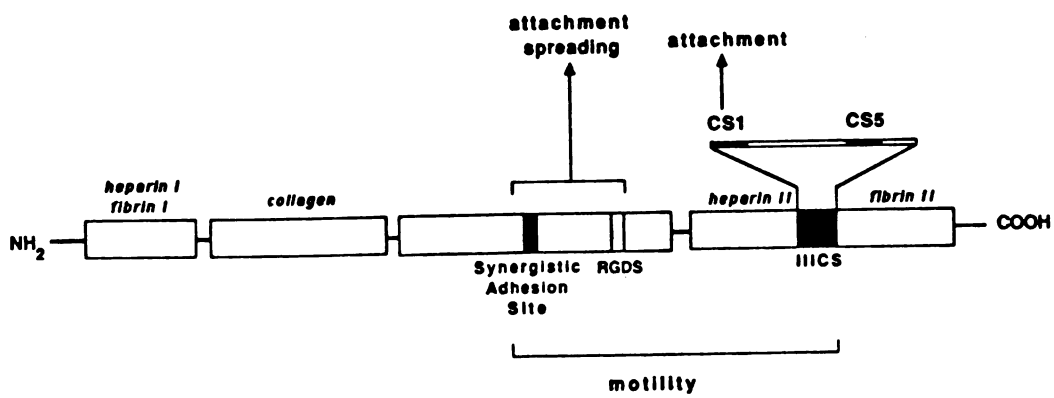


Figure A.2: Attachment of E7 retinal neurons to CS1-KLH after 1 hour as compared to poly-D-lysine (PDL). While insignificant numbers of cells attached to KLH (100 µg/ml coating concentration) alone, 48% attached to CS1-KLH (100 µg/ml). CSAT (100 µg/ml) strongly inhibited attachment to CS1-KLH, whereas the peptide GRGDSP (100 µg/ml) did not have a significant effect. The data reflect the mean and SEM of triplicate determinations.

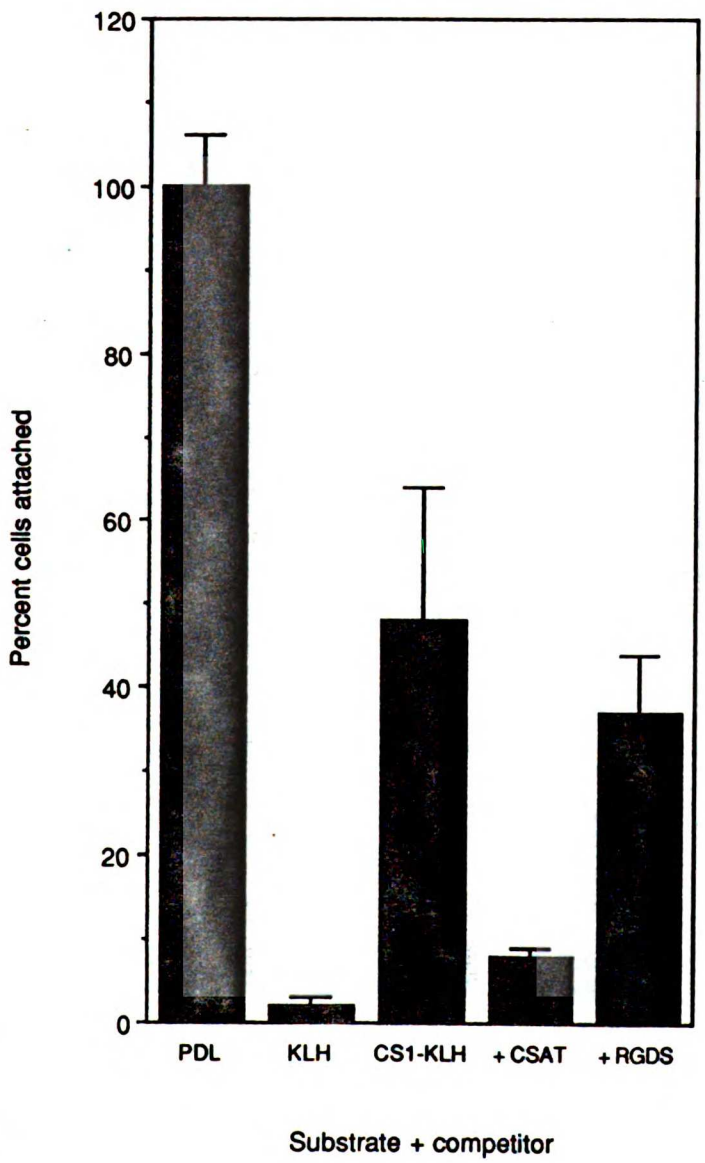
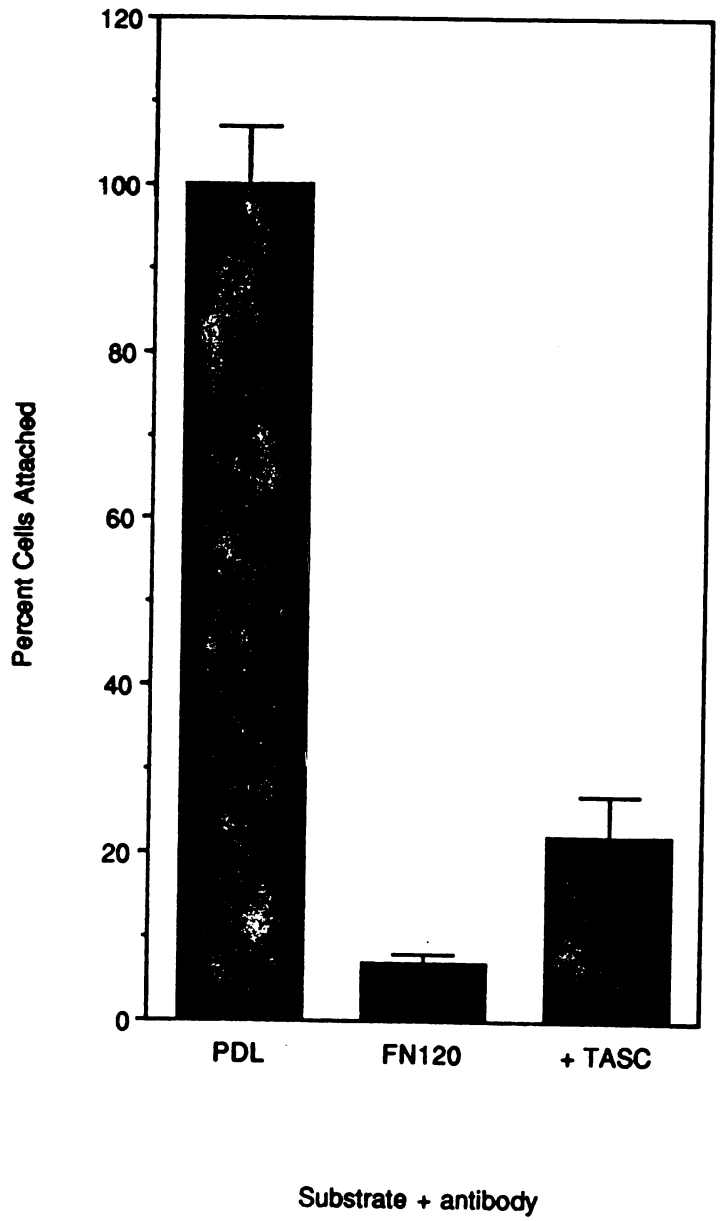


Figure A.3: Attachment of E7 retinal neurons to the 120kD chymotryptic fragment of FN (FN120, 100 µg/ml coating concentration). 7% of the cells as compared to PDL attached to FN120 after 20 min. TASC (100 µg/ml) increased attachment to 22%.



APPENDIX B

Adhesion of a Chicken Myeloblast Cell Line to Fibrinogen Through a β_1-Class Integrin

Abbreviations used in this appendix: FBG, fibrinogen; VN, vitronectin; FN120, 120kD cell-binding fragment of fibronectin; Col, collagen; LPS, lipopolysaccharide.

INTRODUCTION

The integrins are a family of cell surface, heterodimeric receptors that mediate a variety of responses to extracellular ligands including cell adhesion, motility, and the regulation of gene expression (cf. Akiyama, et al., 1990; Hemler, 1990). Each heterodimer is composed of an α and β subunit non-covalently associated with one-to-one stoichiometry, and the particular combination of α and β subunits determines the ligand-binding specificity of the dimer. Our knowledge of α and β subunit diversity and the complexity of dimer function has expanded in recent years to include at least 12 α subunits, 6 β subunits, and a large number of secreted and cell surface ligands. The usefulness of stable cell lines in the study of integrin structure and regulated function has been long been appreciated. Previous to this report, however, no avian cell line which might serve as a model system had been described. Here we report the characterization of the chicken myeloblast cell line HD11 (Beug et al., 1979) as such a system, by identifying the ligands to which they adhere and the integrins that they express.

We have used a panel of function-blocking monoclonal antibodies to examine the roles of β_1-class, α_v-containing, and $\alpha_v\beta_3$ heterodimers in HD11 cell attachment to fibronectin, collagen type I, vitronectin and fibrinogen. Previously described integrin receptors that bind fibronectin include $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ (cf. Akiyama et al., 1990), and $\alpha_v\beta_1$ (Vogel et al., 1990). Binding to collagens has been attributed to $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ heterodimers (cf. Akiyama et al., 1990). Consistent with these precedents for integrin binding specificity, we find that all HD11 cell binding to collagen I and the 120kD chymotryptic cell binding fragment of fibronectin can be attributed to β_1-class integrin receptors. We also provide evidence that β_1-class integrins function in the attachment of HD11 cells to vitronectin and fibrinogen. This is the first report that a β_1-class integrin binds fibrinogen. $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ are known to bind fibrinogen on some cells (Pytela, et al., 1986; Languino et al., 1989). Although one report shows $\alpha_v\beta_1$

dimer binding to vitronectin (Bodary and McLean, 1990), attention has been focussed in recent years on $\alpha_{II}\beta_3$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ function as vitronectin receptors (Pytela et al., 1986; Cheresh and Spiro, 1987; Cheresh et al., 1989). $\alpha_v\beta_3$ also contributes to HD11 cell attachment to vitronectin and fibrinogen, consistent with previous reports of $\alpha_v\beta_3$ binding specificity (Cheresh and Spiro, 1987).

EXPERIMENTAL PROCEDURES

Cells--The HD11 chicken myeloblast cell line (Beug et al., 1979) was provided by Dr. K. Klassing of the University of California (Davis, CA), and cells were maintained under a 5% CO_2 atmosphere in RPMI plus 10% fetal bovine serum, penicillin, and streptomycin. When indicated, cells were stimulated with 5 μg/ml lipopolysaccharide (Sigma, St. Louis) for 24 h before use.

Antibodies--The polyclonal antiserum to the cytoplasmic domain of the β_1 subunit has been described (Tomaselli et al., 1988a). Hybridoma cells secreting the CSAT monoclonal antibody (integrin β_1-specific) were the generous gift of Dr A.F. Horwitz, University of Illinois (Urbana, IL). LM609 ($\alpha_v\beta_3$-specific) ascites fluid was kindly provided by Dr D.A. Cheresh, Research Institute of the Scripps Clinic (La Jolla, CA). The TASC (anti-β_1) and Chav-1 (anti-α_v) monoclonal antibodies were isolated in this laboratory as described (chapters 4 & 5). CSAT, LM609, Chav-1 and TASC IgG's were purified from ascites fluid by 50% ammonium sulfate precipitation, followed by chromatography on Protein A-Sepharose Cl-4B (Pharmacia, Uppsala, Sweden) as per Ey et al. (1978). IgG's were dialyzed against calcium- magnesium-free PBS (CMF-PBS; 200 mg/l KCl, 200 mg/l K_2SO_4, 8 g/l NaCl, and 2.16 g/l $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) and stored at -70°C.

Adhesive Proteins--Vitronectin was purified from fetal bovine serum by chromatography on Heparin Sepharose Cl-6B (Pharmacia, Uppsala, Sweden) as described (Yatogo et al., 1988). Fibrinogen was the generous gift of Dr Z. Ruggeri, Research Institute of the Scripps Clinic (La Jolla, CA). Collagen type I was purchased from Collaborative Research, Inc. (Bedford, MA). The cell-binding, 120kD chymotryptic fragment of fibronectin was obtained from Calbiochem (San Diego, CA).

Cell Adhesion Assays--Cell attachment was measured as previously described in Hall et al. (1987) with the following modifications: Non-tissue culture plastic, 96-well

Linbro Titertek plates (Flow Laboratories, McLean, VA) were coated with 75µl of protein-coating solution. VN and FN120 were diluted to 2 µg/ml and 50 µg/ml, respectively, in CMF-PBS. FBG was diluted to 20 µg/ml in Ca^{++}/Mg^{++}-containing PBS (above solution plus 1mM $CaCl_2$ and 1mM $MgCl_2$). Col I was diluted to 20 µg/ml in 0.1M glacial acetic acid. After overnight incubation at 4°C, each well was incubated with 1% BSA in PBS for 3 hours at 37°C to eliminate non-specific binding. Antibodies and peptides were added to the wells in serum-free medium at twice the final concentration and allowed to equilibrate in the incubator while the cells were being prepared. HD11 cells were harvested with 0.05% trypsin/0.02% EDTA, spun down, and resuspended in RPMI. 100,000 cells were seeded in each well, and the plates were spun at 48xg for 2 min. After incubation at 37°C of varying duration, non-adherent cells were washed from the substrate. Adherent cells were fixed overnight at 4°C in 2.5% glutaraldehyde in PBS, stained with 0.5% crystal violet, solubilized with 1% SDS, and quantitated by A_{540} (Bodary et al., 1989) in a microtiter plate reader (Flow Laboratories). In each experiment, all determinations were done in triplicate.

Immunoprecipitation--HD11 myeloblasts were metabolically labeled with 100 µCi/ml ^{35}S-methionine/cysteine (NEN, Boston, MA) for 16-20 hours. Cells were mechanically removed from their plates with a rubber policeman, spun down, and resuspended in and equal volume of ice-cold Ca^{++}/Mg^{++}-containing PBS plus 1% Triton X-100 and 2mM PMSF. After 15 min of incubation on ice, the extracts were spun at 10,000 rpm in a refrigerated microfuge. The supernatants were divided into 200 ml aliquots, and 20 µg of each antibody was added. Samples were rocked overnight at 4°C and then pre-cleared twice with 50 µl of Sepharose Cl-4b (Pharmacia). Antibody-antigen complexes were precipitated with Protein A-Sepharose Cl-4B (Pharmacia) and washed extensively with 0.05M Tris pH 8.0/ 150 mM NaCl/ 1mM $CaCl_2$/ 1mM $MgCl_2$/ 0.05% Triton X-100. Pellets were extracted in sample buffer and separated by SDS-PAGE on 6% gels (Laemmli, 1970). Gels were fixed, stained, treated with

En^{3}Hance (NEN), dried, and exposed to Kodak X Omat R film (Rochester, NY). Molecular weight standards were from Biorad (Richmond, CA).

RESULTS AND DISCUSSION

As a first step towards characterizing the responses of the chicken myeloblast cell line HD11 to purified extracellular matrix molecules, HD11 cell attachment to the cell binding fragment of fibronectin (FN120), collagen type I (Col I), vitronectin (VN), and fibrinogen (FBG) was measured. As shown in Figure B.1, 100% of the cells attached to FN120 and VN, ~50% attached to Col I, and ~25% attached to FBG in a 40 minute assay. Since treatment of these cells with lipopolysaccharide (LPS) results in their assumption of a macrophage-like phenotype (Beug et al., 1979), it seemed possible that their responses would change with LPS treatment. LPS-stimulated HD11 cells continued to attach well to FN120, but attachment to Col I, VN, and FBG was significantly decreased (Fig. B.1). Neither LPS-stimulated nor unstimulated HD11 cells attached to laminin (data not shown). In the studies that follow, only unstimulated HD11 cells were used, since these were generally more adhesive. However, the effects of LPS stimulation on the adhesion of HD11 cells to different substrates indicates that this cell line will provide a useful system for future studies on the regulation of the expression and activity of extracellular matrix receptors.

To identify receptors that mediate attachment to these purified glycoproteins, we used a panel of function-blocking monoclonal antibodies against members of the integrin family of extracellular matrix receptors. These include CSAT (Buck et al., 1986) and TASC (chapter 4) to the integrin β_1 subunit, Chav-1 (chapter 5) to the integrin αv subunit, and LM609 to the $\alpha_v\beta_3$ complex (Cheresh, 1987). Immunoprecipitation analysis showed that HD11 cells express all three antigens (Fig. B.2). CSAT, TASC, and a polyclonal antibody against the cytoplasmic domain of the β_1 subunit all precipitated the β_1 subunit at 120kD in association with at least three apparent α subunits of Mr 160, 145, and 140k from metabolically labeled cells (Fig. B.2, lanes 1-3). A 100kD band of variable intensity was observed upon precipitation of β_1-class integrins. Since two bands at 120 and 100kD were detected by TASC in

immunoblots of HD11 cell extracts (chapter 4), the 100kD protein probably represents a degraded or precursor form of the β_1 subunit. LM609 (lane 4) immunoprecipitated two bands, α_v at 150kD and β_3 at 95kD. Chav-1 (lane 5) and a polyclonal antibody against the cytoplasmic domain of the α_v subunit (data not shown) precipitated a similar pattern of proteins, except that the lower band was more prominent and broader (95-100kD). α_v has been shown to dimerize with β_5 which is resolved on non-reducing gels at a molecular weight 2-5kD heavier than β_3 (Cheresh et al., 1989; Freed et al., 1989). Thus, it is possible that the 95-100kD band immunoprecipitated with Chav-1 contains β_5 in addition to β_3. α_v has also been shown to dimerize with b_1 (Bodary et al., 1990; Vogel et al., 1990; Bossy and Reichardt, 1990) in addition to β_3 and β_5, but this heterodimer is apparently not a major species expressed by HD11 cells, since a 120kD band was not co-precipitated with the α_v subunit.

Attachment to FN120 and Col I was studied, using the above antibodies and the arg-gly-asp-containing hexapeptide, GRGDSP (Pierschbacher and Ruoslahti, 1984), to perturb integrin function. The strong inhibitory effects of the integrin β_1-specific mAb CSAT on HD11 cell attachment to FN120 (Fig. B.3A) and Col I (Fig. B.3B) indicate that HD11 cell interactions with both ligands can be attributed to the action of integrin β_1-class heterodimers. In addition, the RGD peptide effectively inhibited attachment to FN120 but not to Col I. The integrin β_1-specific mAb TASC has been shown to enhance cell binding mediated by β_1-class integrins to many ligands (chapter 4) and did so in these experiments, increasing attachment to FN120 2-fold and to Col I 2.7-fold. Consistent with the proposal that the TASC antibody specifically promotes the activity of

β_1-class integrins, another β_1-specific mAb CSAT blocks all of the attachment measured in the presence of TASC. Neither α_v (Chav-1) nor $\alpha_v\beta_3$ (LM609) antibodies inhibited attachment to FN120 or to Col I, indicating that the α_v-containing heterodimers expressed by these cells did not bind to either ligand. The lack of

inhibition by anti-α_v on FN120 and Col I also makes it unlikely that α_v in association with a β_1 subunit variant accounts for binding to either ligand as has been recently reported in studies of a neuroblastoma cell line (Dedhar and Gray, 1990).

HD11 cell attachment to VN was almost completely inhibited by the RGD peptide, indicating that integrins mediate attachment of these cells to VN (Fig. B.4). Additional data presented in Figure B.4 shows that binding to VN reflects the activity of at least two different integrin heterodimers, $\alpha_v\beta_3$ and a β_1-containing heterodimer. Although TASC antibody promotes β_1-class integrin-mediated cell attachment to other substrates, it was isolated for its ability to inhibit attachment to VN (chapter 4). The β_1-specific monoclonal antibodies CSAT and TASC inhibited HD11 cell attachment to VN by 42% and 59% respectively, and the combination of CSAT plus TASC reduced attachment by 85% of control (Fig. B.4). The additive behavior of these two antibodies indicates that either two β_1-class integrin heterodimers act as VN receptors on these cells or that both function as partial inhibitors of the same heterodimer. We cannot yet distinguish between these possibilities nor do we know the identity of the associated a subunit(s). Buck and Horwitz (1987) have also reported the binding of chicken embryo β_1-class integrins to VN, but have not identified the a subunit active in their system. A recent report (Bodary and McLean, 1990) describes the function of $\alpha_v\beta_1$ in the attachment of the human embryonic kidney cell line 293 to VN; others find $\alpha_v\beta_1$ on the human neuroblastoma cell line IMR32 mediates cell attachment to FN rather than VN (Vogel et al, 1990). Because we were unable to detect the β_1 subunit in immunoprecipitates from HD11 cells using α_v-specific antibodies, it seems unlikely that $\alpha_v\beta_1$ is responsible for β_1 integrin-mediated attachment of HD11 cells to VN.

The α_v-specific monoclonal antibody Chav-1 also reduced attachment to VN, by 80% (Fig. B.4), indicating the function of an α_v-containing heterodimer(s) in VN binding. $\alpha_v\beta_3$ is likely to account for some of this activity, since it is expressed by these cells (see Fig. 2) and since the $\alpha_v\beta_3$-specific antibody LM609 had significant,

though weaker, effects on cell attachment (Fig. B.4). While LM609 alone inhibited attachment to VN by only 25%, the combination of CSAT and LM609 was more effective, reducing attachment by 75%. The residual attachment to VN and the strong effects of Chav-1 combine suggest that either Chav-1 is a better competitor of $\alpha_v\beta_3$ function or that another α_v-containing heterodimer (eg. $\alpha_v\beta_5$) is expressed by these cells. Indeed, $\alpha_v\beta_5$ has been detected on primary human monocytes which also express $\alpha_v\beta_3$ (Krissansen, et al., 1990).

HD11 cell attachment to FBG was RGD-dependent and, like VN, appeared to depend on the activity of both β_1- and α_v-containing integrin heterodimers (Fig. B.5). Whereas the β_1-specific mAb CSAT completely blocked attachment to FBG, the TASC mAb against β_1 promoted attachment 2.3-fold. The fact that these results were obtained in a 5 minute assay (~10 minutes from the time of trypsinization) argues against the possibility that the HD11 cells secrete a β_1-class integrin ligand (eg. FN) that was adsorbed to the BSA-blocked plastic surface or to FBG itself. Chav-1 reduced attachment to FBG by 80% and the $\alpha_v\beta_3$-specific mAb LM609 completely blocked attachment (Fig. B.5). Again, this result makes it unlikely that the substrate had been conditioned by HD11-derived β_1-class ligands, since attachment to none of the other ligands tested was so effectively inhibited by LM609. Thus, both $\alpha_v\beta_3$ and β_1 associated with an unknown α subunit appear to play critical roles in HD11 cell attachment to FBG, since LM609, CSAT and TASC each abolished all cell binding. The observation that Chav-1 did not significantly reduce TASC-enhanced attachment suggests that α_v is not the β_1-associated α subunit. This is consistent with our failure to detect $\alpha_v\beta_1$ heterodimer expression by these cells (see Fig. B.2). Interestingly, LM609 did not inhibit TASC-enhanced FBG binding, suggesting that increased function of the β_1-class FBG receptor is sufficient to support cell attachment in the absence of $\alpha_v\beta_3$ function.

To summarize (Table B.1), we have presented evidence for a novel mechanism

for cell interaction with FBG which is mediated by an integrin heterodimer composed of the β_1 subunit in association with an unknown α subunit. It is unlikely that α_v is the subunit in question, since the chicken myeloblast HD11 cells used in these studies do not express detectable levels of $\alpha_v\beta_1$. Moreover, Ghav-1, a function-blocking monoclonal antibody against the chicken α_v subunit did not inhibit FBG binding when β_1-class integrin function was enhanced by the monoclonal antibody TASC. Not all cells express this receptor, since embryonic chick retinal neurons did not attach to FBG even in the presence of TASC (chapter 4). Further experiments are required to identify the $\alpha\beta_1$ dimer that binds FBG on HD11 cells. In addition, we provide evidence that the $\alpha_v\beta_3$ heterodimer also functions as a FBG and VN receptor on HD11 cells, consistent with the binding specificity reported for $\alpha_v\beta_3$ by others (Cheresh and Spiro, 1987). We have not tested HD11 cell binding to von Willebrand's Factor, another $\alpha_v\beta_3$ ligand for melanoma cells (Cheresh and Spiro, 1987). Interestingly, $\alpha_v\beta_3$ functions on primary human macrophage to mediate the specific killing of dying neutrophils (Savill et al., 1990), raising the possibility that $\alpha_v\beta_3$ has a cellular ligand as well. The HD11 cell line may provide a useful probe for the identification of such a ligand.

HD11 cell attachment to FN120 and Col I was found to be entirely β_1-dependent. The prominent 160kD band that co-precipitates with β_1 from these cells is likely to represent the α_5 subunit, since cell attachment to FN120 is sensitive to arg-gly-asp-containing peptide and since anti-α_5 antisera immunoblot a band at this molecular weight from HD11 cell extracts and from whole chick embryos (K. Venstrom and L.F. Reichardt, unpublished results; Hynes et al., 1989). The β_1-class heterodimer(s) responsible for attachment to Col I may include $\alpha_3\beta_1$ which has been shown to function as a collagen receptor, since anti-α_3 antisera recognize a band at the appropriate molecular weight (140kD) in immunoblots of HD11 proteins (K. Venstrom and L.F. Reichardt, unpublished results; Hynes et al., 1989). The observation that LPS stimulation of these cells decreases their responsiveness particularly to Col I (see Fig.

B.1) provides a compelling reason to identify and further study this heterodimer(s). Recent reports have described the activation-dependent increase in β_1-class integrin laminin receptor function on primary murine macrophages and human lymphocytes (Shaw et al., 1990; Shimizu et al., 1990). In these studies, integrin activation is associated with changes in levels of receptor phosphorylation and cytoskeletal association (Burn et al., 1988; Shaw et al., 1990). The observed *reductions* in integrin receptor function upon LPS treatment, especially on collagen, suggest that the HD11 cell line will be a convenient model system for studies of the downregulation of receptor function which may be due to different regulatory mechanisms acting on a distinct set of integrin heterodimers.

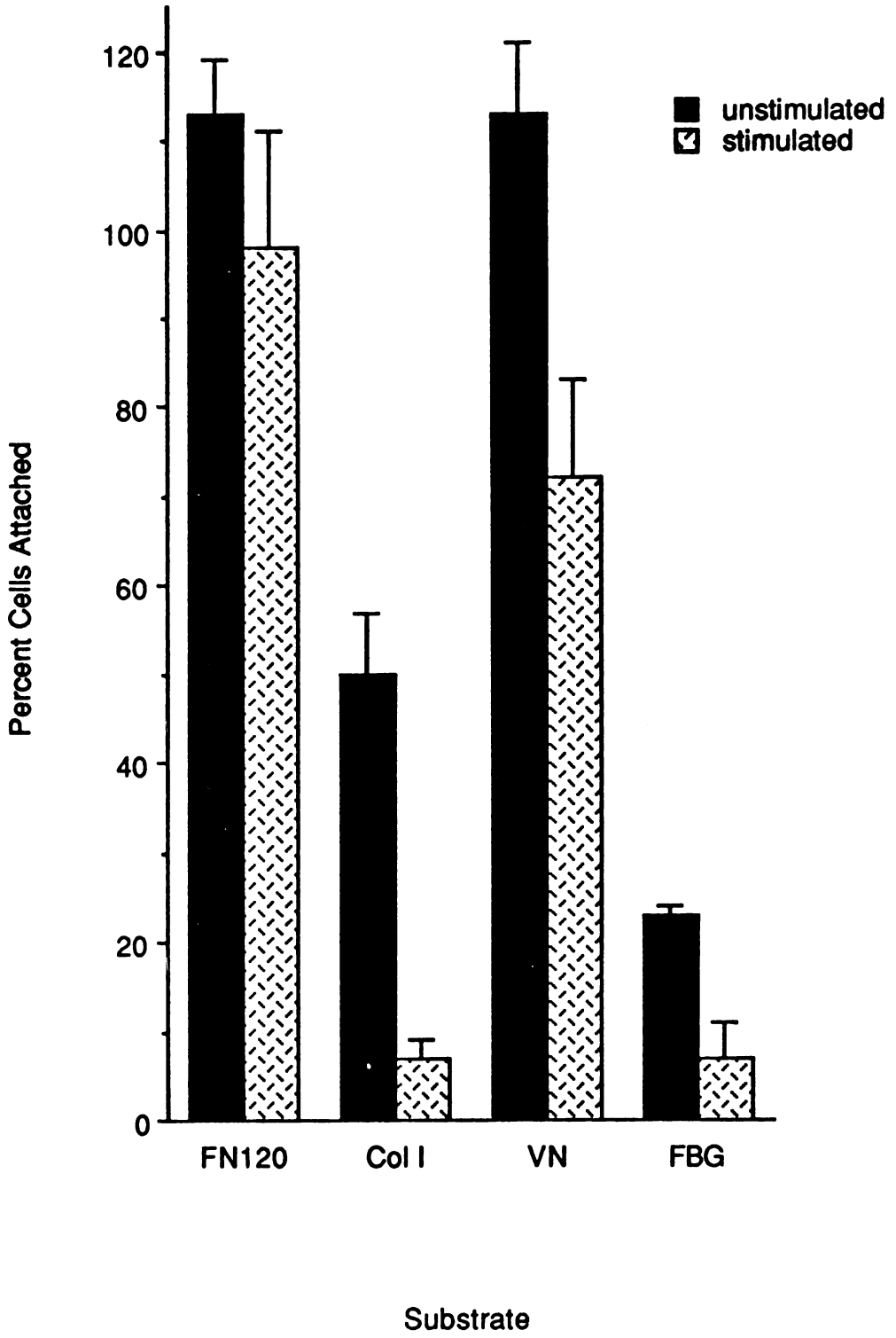
Table B.1: Summary of the integrin receptors involved in HD11 cell attachment to FN120, collagen I, vitronectin, and fibrinogen. See text for results and rationale.

Table 1. Summary of HD11 cell receptor interactions with various ligands.

Ligand	Integrin Receptors(s)
Fibronectin-120	all β_1-class
Collagen type I	all β_1-class
Vitronectin	$\alpha_V\beta_3$, $\alpha_V\beta_?$, $\alpha_?\beta_1$
Fibrinogen	$\alpha_V\beta_3$ and $\alpha_?\beta_1$

Figure B.1: Attachment of HD11 cells to FN120, Col I, VN, and FBG with (stippled bars) and without (filled bars) prior stimulation by LPS. Attachment to each substrate is normalized to that measured on poly-D-lysine, taken to represent 100%. Each determination was made in triplicate, and values represent the mean +/- SEM.

Figure 1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100
80
60
40
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Percent Cells Attached
unstimulated
stimulated
FN120
Col I
VN
FBG
Substrate

Figure B.2: Immunoprecipitation of HD11 integrins visualized by autoradiography. ^{35}S-methionine/cysteine labeled HD11 cells were extracted and immunoprecipitated with (lane 1) a polyclonal antiserum against a cytoplasmic peptide predicted from the sequence of the human b_1 subunit (Tomaselli et al., 1988a), (2) CSAT, (3) TASC, (4) LM609, and (5) Chav-1. Antigen/antibody complexes were resolved by SDS-PAGE under non-reducing conditions on 6% gels.

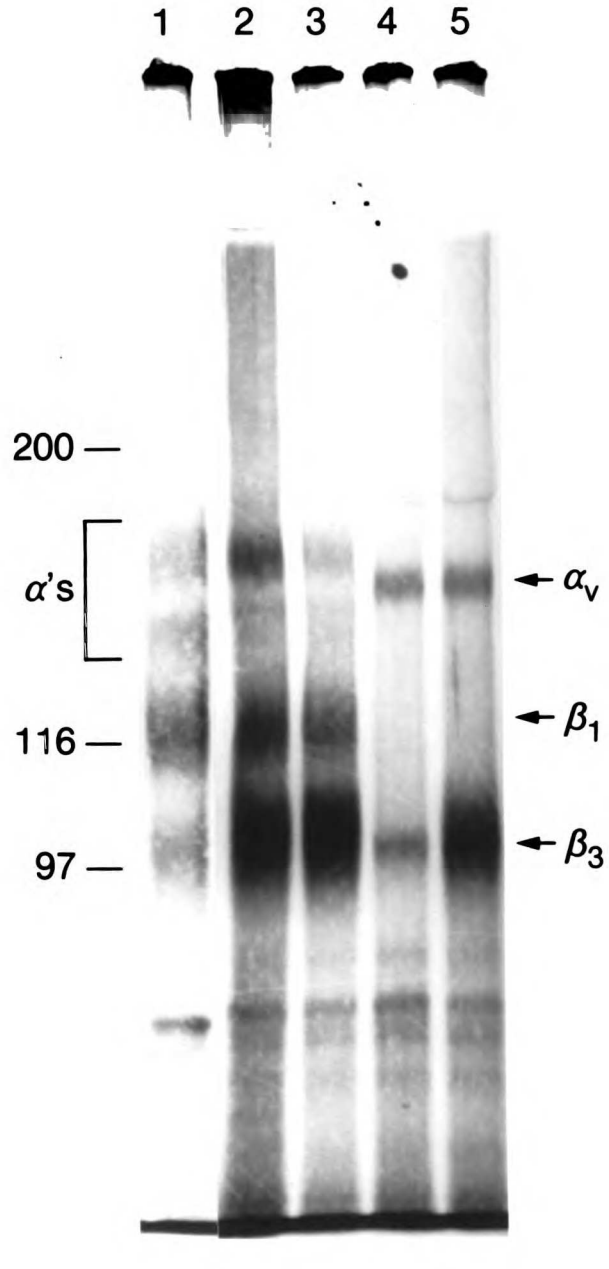

1
2
3
4
5
200
α's
116
97
α_v
β_1
β_3

Figure B.3: Effects of function-blocking monoclonal antibodies and the GRGDSP peptide on HD11 cell attachment to FN120 in 5 minutes (a) and Col I in 40 minutes (b). Abbreviations: O, no antibody added; R, 100 μg/ml GRGDSP; C, 50 μg/ml CSAT IgG; T, 200 μg/ml TASC IgG; CT, 50 μg/ml CSAT IgG plus 200 μg/ml TASC IgG; V, 100 μg/ml Chav-1 IgG; L, 100 μg/ml LM609 IgG. Each determination was made in triplicate, and values represent the mean +/- SEM normalized to the attachment measured in the absence of antibody.

Figure 3a

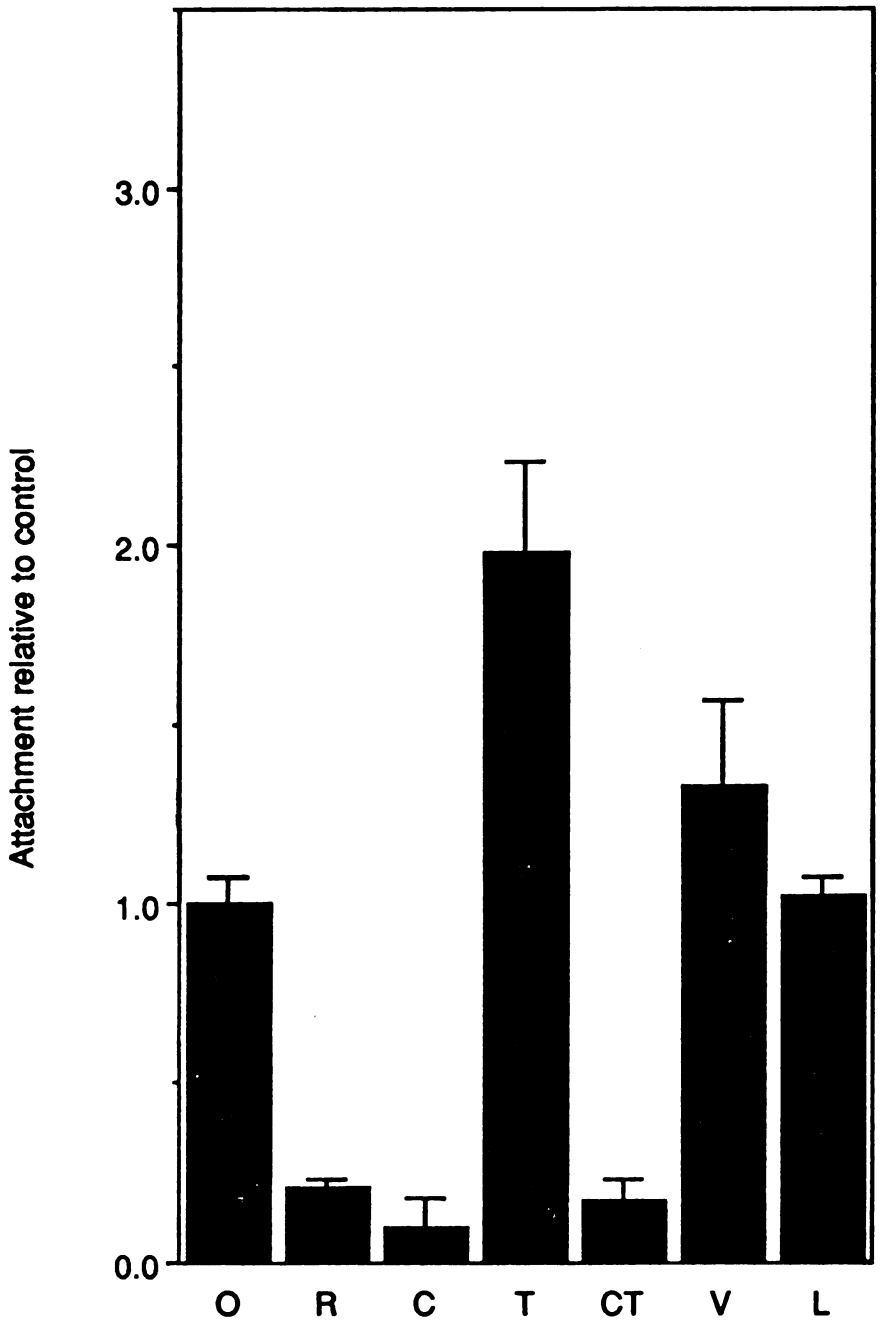
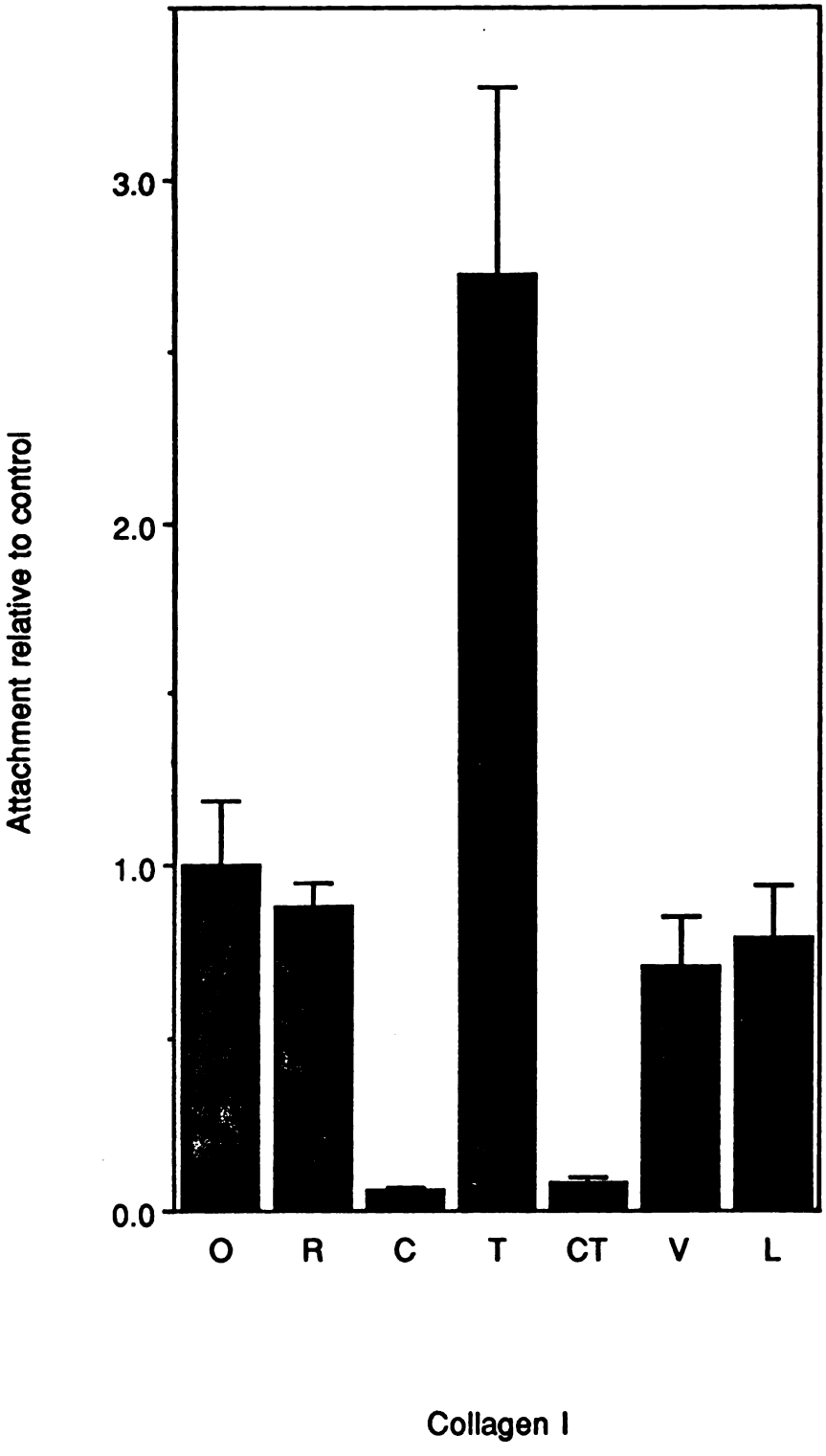


Figure 3b


Attachment relative to control
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R
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T
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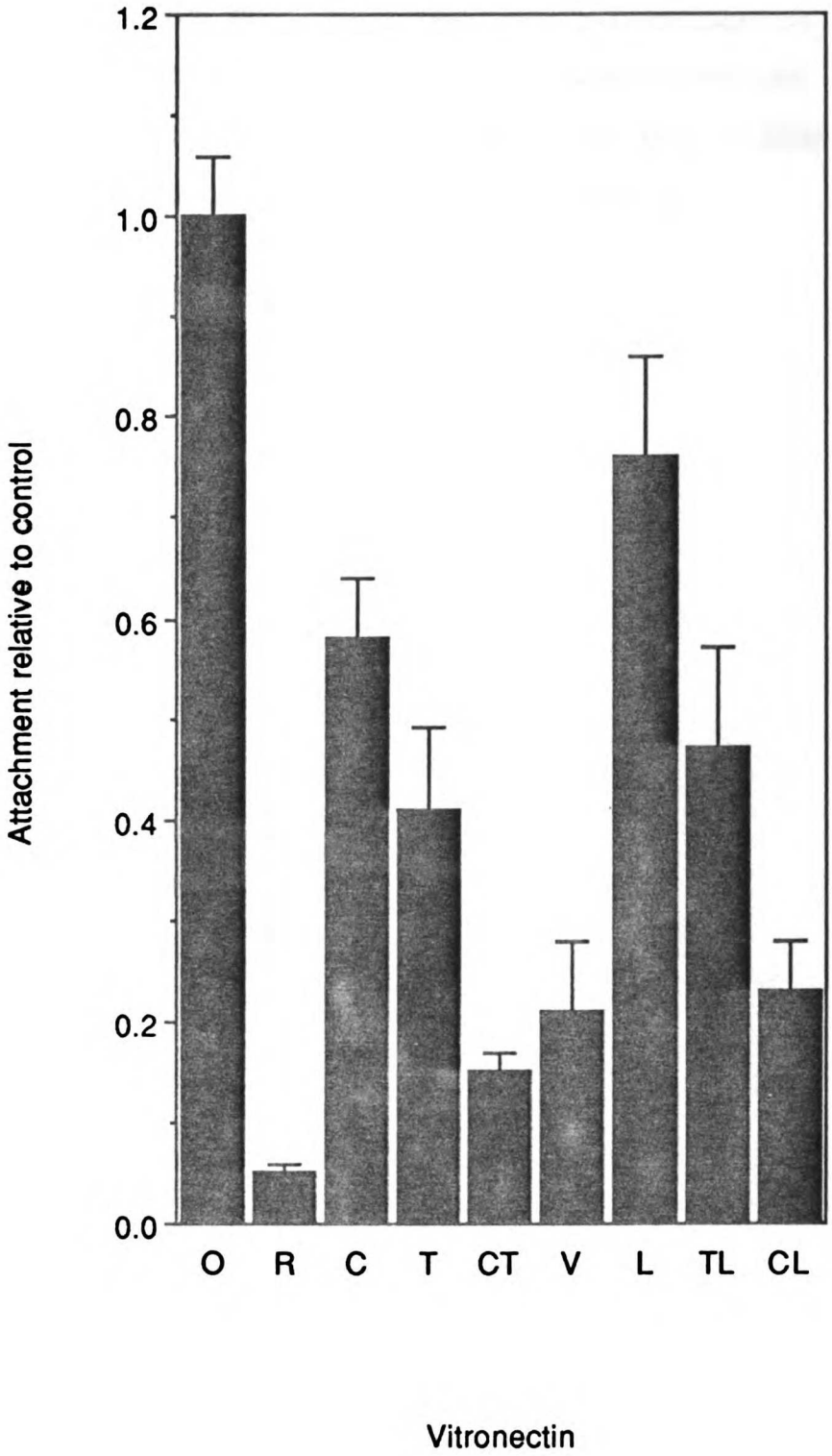
V
L
Collagen I

Figure B.4: Effects of function-blocking monoclonal antibodies and the GRGDSP peptide on HD11 cell attachment to VN in 5 minutes. Abbreviations as in Fig. 3., except TL, 200 μg/ml TASC IgG plus 100 μg/ml LM609 IgG; CL, 50 μg/ml CSAT IgG plus 100 μg/ml LM609 IgG. All values are normalized to the attachment measured in the absence of antibody (O). Each determination was made in triplicate, and values represent the mean +/- SEM normalized to the attachment measured in the absence of antibody.

Figure 4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0.8
0.6
0.4
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Attachment relative to control
O
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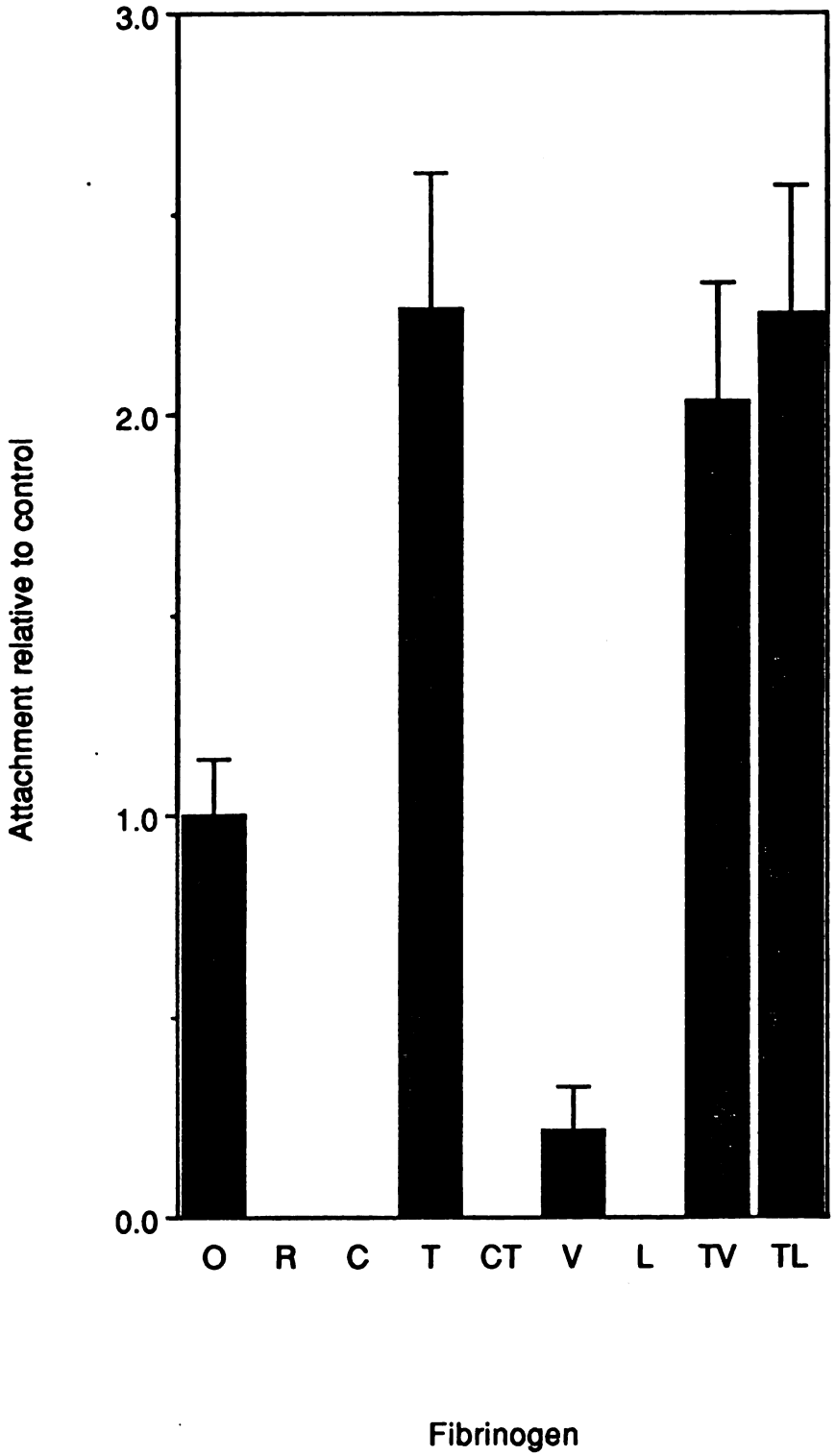
TL
CL
Vitronectin

Figure B.5: Effects of function-blocking monoclonal antibodies and the GRGDSP peptide on HD11 cell attachment to FBG in 5 minutes. Abbreviations as in Fig. 3., except TV, 200 μg/ml TASC IgG plus 100 μg/ml Chav-1 IgG. All values are normalized to the attachment measured in the absence of antibody (O). Each determination was made in triplicate, and values represent the mean +/- SEM normalized to the attachment measured in the absence of antibody.

Figure 5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