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Chapter 31

Extracellular Matrix in the Developing Nervous System

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INTRODUCTION

Extracellular matrix (ECM), in its broadest definition, refers to all the molecules that are immobilized in the spaces between cells. The most extensively studied ECMs are those found in connective tissues, such as bone, cartilage, tendons, ligaments, fascia, and the stromal components of organs. Connective tissues are typically cell-poor, i.e., the ECM comprises a major fraction of the tissue mass. Accordingly, connective tissue ECMs are comparatively easy to isolate in large quantities, permitting direct biochemical analysis.

In contrast, the ECMs of nonconnective tissues are difficult or in some cases impossible (so far) to isolate, and usually must be studied by more indirect means. For this reason most progress toward an understanding of nonconnective tissue ECMs has been made only in the last 15–20 years. Under the circumstances, it is not surprising that most generalizations that have been made about how ECMs are organized and how they function have been drawn from work on connective tissues, and are not necessarily applicable to the ECMs of nonconnective tissues. Nowhere is this fact more evident than when considering the ECM of the central nervous system (CNS). For example, it is traditional to think of ECMs as consisting largely of structural molecules, such as collagens and elastin, that confer bulk tissue properties such as resistance to stress, rigidity, elasticity, or compressibility. The CNS, however, is virtually devoid of these molecules (except in association with meninges and blood vessels), a fact that explains its jellylike consistency. Moreover, while it is traditional to expect ECMs to take up certain light-microscopic stains and to exhibit certain ultrastructural features (fibrils, electron-dense material), the ECM of the CNS shows virtually none of these characteristics.

Indeed, were it not for the fact that the presence of many known ECM molecules can be readily demonstrated in the brain, there would be serious debate as to whether an ECM existed there at all (as, in fact, there was for many years). Yet the existence of an ECM in the brain underscores the fact that ECMs must have functions beyond the structural and mechanical roles envisaged for them in connective tissues. Indeed, the explosion of research in the ECM field over the past 15 years has focused largely on the nonstructural properties of ECM molecules. The picture that has emerged (and is still changing) is of the ECM as a key regulator of virtually all aspects of cell behavior, including cell proliferation, shape, motility, cell–cell interactions, growth factor responses, even gene expression. During embryonic development, the ECM is uniquely suited, by virtue of its immobility, to being a repository of morphogenetic information, such as the information that guides growing axons to their targets. During carcinogenesis, the ECM is ideally suited to regulate growth, differentiation, invasion, and metastasis.

Most of this new information about ECMs is being pieced together from studies of many types of cells, normal and transformed, *in vivo* and *in vitro*. Although cells of the nervous system have played an important role in this work, some of what is said in this chapter has had to be extrapolated from work on nonneural tissues. Clearly, our current knowledge of the ECM of the nervous system and its functions is at a very early stage, and much new information will undoubtedly be obtained in the next several years. Nevertheless, there is enough information now to conclude that the ECM plays a critical role in the formation of the nervous system, and its response to injury.

FORMS OF ECM IN THE NERVOUS SYSTEM

As stated above, cells of the CNS find themselves surrounded by an ECM that is very different from that of connective tissues. Those connective tissue elements that are present in the CNS are associated with meninges or

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surround blood vessels. Astrocytic processes that end on these structures apparently isolate them from direct contact with other CNS cells. This arrangement also isolates CNS cells from another type of ECM, the basement membrane (or, in the parlance of electron microscopists, the basal lamina). Basement membranes are specialized sheets of ECM almost universally found adjacent to the basal surfaces of epithelia (including the endothelial cells of blood vessels), as well as in a few other locations. During early development, cells and axons may be in contact with the basement membrane that surrounds the neural tube (originally an epithelium), but such contacts are not present in the mature brain.

In contrast to the cells of the CNS, cells of the peripheral nervous system (PNS) do contact conventional matrices, especially basement membranes, which surround Schwann cells and muscles. During peripheral nerve regeneration, axons come into direct contact with these basement membranes. During development, neural cells and axons also come into wide contact with interstitial ECMs, i.e., ECMs that will eventually become components of connective tissues. Thus, the PNS and CNS possess distinctly different ECMs. The change from one type to another occurs abruptly at the glia limitans.

MOLECULES OF THE NERVOUS SYSTEM ECM

Laminin

The term *laminin* was coined to refer to a molecule isolated from a murine tumor (the Engelbreth-Holm-Swarm, or EHS, sarcoma) that produces large amounts of basement membranes (for review, see Beck et al., 1990). This molecule is a large glycoprotein (~850 kd), consisting of three polypeptide chains, one long ("A," ~400 kd) and two shorter, related chains ("B1" and "B2," ~220 and ~210 kd, respectively). All three chains are encoded by separate genes. The organization of the chains into an intact molecule is shown in Figure 1. Like many of the large glycoproteins found in ECMs, laminin consists of many distinct structural motifs strung together. Some of these structural elements have been associated with the ability to bind certain cellular receptors, or have certain effects on cell behavior, especially the behavior of neurons (see below).

Polyclonal antibodies raised against laminin have been used to localize it in many tissues. The results imply that laminin is a ubiquitous and abundant component of basement membranes, and occasionally a much less abundant component of other ECMs. Usually, when laminin is found outside basement membranes its presence is only transient, i.e., it appears only during development, or as part of an injury response. Transiently expresssed laminin is known to be encountered by nervous system cells in several circumstances: In the CNS, laminin appears transiently along a small number of axon tracts, at or about the time that axons are navigating these pathways. Examples include the avian and rodent optic tract and the early-forming ventral longitudinal tract of the mouse hindbrain (Cohen et al., 1987; Letourneau et al., 1988). Laminin is also reexpressed in the CNS at sites of injury (Liesi et al., 1984). In the PNS, non-basement membrane laminin is also found at locations where developing axons grow (e.g., Rogers et al., 1986).

Recently, it has become clear that several isoforms of laminin exist. In these molecules, one or more of the chains of laminin is substituted with a novel, but related, polypeptide. Thus, the "A" chain may be replaced by a homologous "M" chain to generate a molecule referred to as merosin. The "B1" chain may be replaced by a homologous "S" chain to generate what has been called s-laminin. Since most early immunohistochemical studies on the localization of laminin *in vivo* did not use antibodies specific for individual chains, one cannot conclude from such studies which laminin isoforms are present. More recent studies, however, indicate that laminin isoforms have distinctly different distributions *in vivo*. Merosin and classical laminin, for example, are found in different types of basement membranes (Sanes et al., 1990). In the neuromuscular system, laminins containing the "S" chain are specifically localized to the ECM of the neuromuscular junction (the term s-laminin is short for synapse-specific laminin). The form of laminin present in the developing and injured CNS has yet to be determined.

Fibronectin

Fibronectin has probably been more intensively studied than any other ECM protein (for reviews, see Hynes, 1990; Yamada, 1990). Fibronectin is a ~440 kd glycoprotein that is composed of two disulfide-linked polypeptide chains (Fig. 1). Both chains are encoded by a single gene, but are not necessarily identical in amino acid sequence, as considerable alternative splicing of fibronectin transcripts occurs. Alternative splicing patterns are known to be both developmentally regulated and tissue-specific *in vivo*. At least some of the heterogeneity created by alternative splicing affects the ability of fibronectin to interact with certain cellular receptors and to form higher-order fibronectin aggregates and fibrils.

Fibronectin is an abundant component of many connective tissue ECMs, and sometimes may also be found in basement membranes. Fibronectin is also present in soluble form in plasma. In the PNS, fibronectin is widely distributed among matrices through which neural crest cells migrate, as well as in territories encountered by peripheral nerves. In the CNS, fibronectin can be found associated with meninges and blood vessels, but, like laminin, is otherwise expressed only transiently, and in a limited number of sites. Its localization in the brain has been best worked out in the forming mammalian cerbral cortex (Sheppard et al., 1991; Stewart

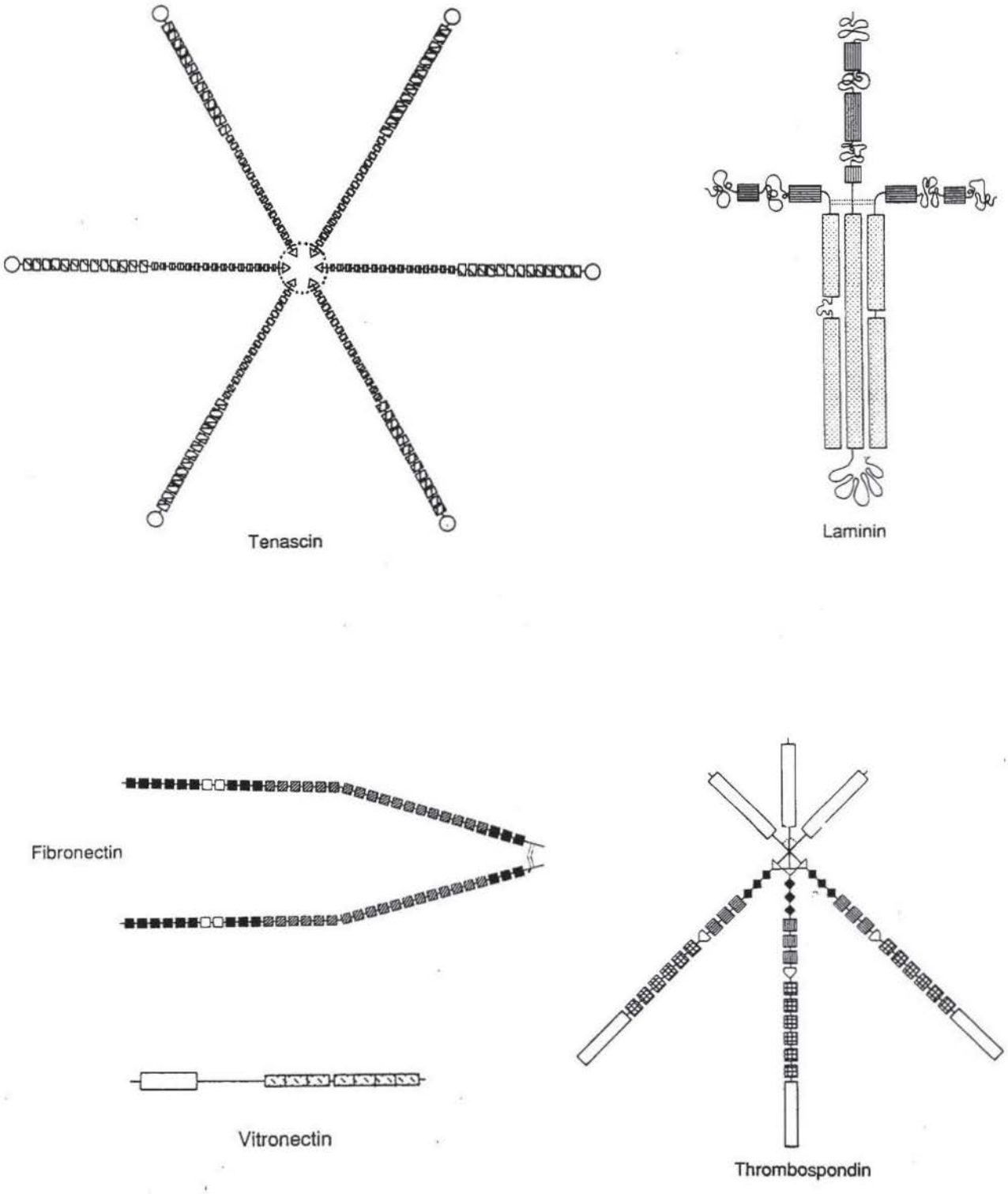

Fig. 1. Structures of ECM proteins found in the central nervous system. These schematic drawings illustrate the polypeptide components and repeating domain structures of five of the best understood ECM components in the nervous system. The rectangular symbols distributed along each polypeptide chain represent repeated amino acid motifs such as EGF-like repeats and fibronectin-type III repeats. Disulfide linkages between polypeptide chains are represented as dotted lines (for further details, see review by Lander, 1989).

and Pearlman, 1987). There it appears earliest in the ventricular zone, where neural precursors proliferate. Later, expression shifts to the subplate region, the layer of developing cortex into which cortical efferent and afferent fibers grow. As the cortex matures, more superficial neuron layers are added, and thalamocortical axons grow out from the subplate and innervate their final targets in the cortex. At about the same time, fibronectin immunoreactivity in the subplate disappears (as does the subplate itself shortly thereafter).

Tenascin

Tenascin, like fibronectin, is a large molecule comprised of identical (or closely related) polypeptide chains held together near their ends by disulfide bonds (for reviews, see Chiquet, 1989; Erickson and Bourdon, 1989). Typically, six polypeptides are linked together to form a six-armed structure known as a hexabrachion (Fig. 1), although forms with fewer arms have been observed in the electron microscope. Tenascin subunits are approximately 190–220 kd in size, and, like fibronectin subunits, vary in structure due to alternative splicing.

Tenascin was discovered more recently than fibronectin and laminin, and was found independently by a number of laboratories. Some of these groups first identified tenascin in nervous system tissue, and some of the early names given to this molecule, such as "cytotactin" and "J1," still persist in some of the neurobiological literature. Although the highest levels of tenascin in the body are found in connective tissue and other mesenchymal ECMs, the developing CNS also contains a considerable amount, especially during late developmental stages, in a wide variety of locations. In laminating CNS structures, such as the developing retina and cerebellum, tenascin can be found associated with particular cell layers and with the radial glial cells that are thought to guide cell migration into appropriate layers. In some CNS structures, such as the developing rodent somatosensory cortex, tenascin expression is transiently associated with the boundaries that separate functional units (such as "barrels"). In the developing PNS, tenascin is strongly associated with the pathways followed by neural crest cells, and later with peripheral nerves and ganglia.

Although the expression of tenascin in the developing CNS is much more widespread than that of laminin and fibronectin, it too largely disappears from the mature brain. The strong expression of tenascin by human glioma cells, however, suggests that reexpression of tenascin may be an important concomitant of neoplastic transformation. Indeed, tenascin is a prominent component of many neural and nonneural tumors. Among human gliomas, tenascin expression tends to correlate with a poorly differentiated histological appearance (Erickson and Bourdon, 1989).

Thrombospondin

Like fibronectin and tenascin, thrombospondin is a large (~420 kd) glycoprotein composed of multiple similar subunits (Lawler and Hynes, 1986). Three polypeptide chains are disulfide-bonded together to form an intact thrombospondin molecule (Fig. 1). Currently, the three chains in each molecule are thought to be identical, but the possibility of alternative splicing to produce molecular heterogeneity has not been ruled out. Recently, the existence of a second gene that encodes a thrombospondin isoform was reported (Bornstein et al., 1991). It is not yet known whether the product of this gene accounts for any of the thrombospondin that has been biochemically or immunohistochemically identified so far, nor whether thrombospondin trimers exist that contain a mixture of chains from the two thrombospondin genes.

Thrombospondin was first identified as a molecule released by activated platelets, and was later found to be a major ECM protein secreted by endothelial and smooth muscle cells. Although thrombospondin secretion by cultured human glial cells was noted relatively early (Asch et al., 1986), full appreciation of the widespread expression of thrombospondin in the nervous system awaited the development of antibodies that could be used on rodent and avian tissue. It is now known that thrombospondin appears early and abundantly througout the developing CNS. In the developing cerebellum, thrombospondin appears transiently in particular layers (O'Shea et al., 1990). Unlike laminin, fibronectin, and tenascin, thrombospondin remains expressed at high levels in many parts of the CNS, suggesting that thrombospondin may play a role in mature brain functions.

Vitronectin

Vitronectin is an ECM glycoprotein that, like fibronectin, is present at relatively high levels in plasma. It is an unusually small ECM component, consisting of a single polypeptide chain of ~78 kd (Fig. 1). Because of vitronectin's presence in plasma, immunolocalization studies must be carefully controlled to rule out artefactual staining arising from vascular leakage. So far, only one region of the CNS, the developing retina (along with the optic nerve), has been shown to contain vitronectin (Neugebauer et al., 1991). Whether vitronectin persists in this location in the mature brain, and whether it is present elsewhere in the CNS, remains to be determined.

J1-160/180

Among the many groups to independently identify tenascin (see above), one isolated a monoclonal antibody, termed "J1," that additionally immunoprecipitates something else from mouse brain. This additional material is composed of

polypeptide chains of 180 and 160 kd and is clearly immunologically distinguishable from tenascin (for discussion, see Morganti et al., 1990). Like tenascin, however, electron microcgraphs of J1-160/180 preparations reveal multi-armed structures (usually one, two, or three arms, as opposed to tenascin's six) attached at a central point. Based on these facts, and some of the biological activities of J1-160/180 (see below), it is reasonable to speculate that J1-160/180 will turn out to be structurally related to tenascin. Clarification of this issue currently awaits molecular cloning efforts.

Despite the apparent relatedness of J1-160/180 to tenascin, its tissue distribution is quite different. In the rodent, J1-160/180 appears only after birth, and is found only in white matter of the CNS, where it is associated with oligodendrocytes and the myelin they produce. J1-160/180 appears not to be expressed outside the nervous system, an almost unique characteristic for an ECM molecule.

Agrin

The discovery and characterization of agrin were the result of a concerted effort to identify a molecule involved in neuromuscular synaptogenesis. Although muscle cells are surrounded entirely by an ECM (a basement membrane or basal lamina), the matrix lying between nerve and muscle at synapses has long been known to contain unique components and possess distinctive biological activities (reviewed by Sanes, 1989). Intriguingly, the synaptic basal lamina alone is capable of causing muscle to assemble a postsynaptic apparatus. One of the most striking features of this apparatus is a very dense clustering of cell-surface acetylcholine receptors. Through a series of investigations, a molecule, agrin, was identified that could trigger acetylcholine receptor clustering *in vitro*. Recent molecular cloning experiments indicate that that molecule is a fragment of a large (~215 kd) ECM protein that contains regions of amino acid similarity to laminin and to certain protease inhibitors (Rupp et al., 1991). *In situ* hybridization experiments indicate that agrin is expressed in the developing nervous system—especially by spinal motoneurons—as well as the developing gut and aorta. Antibody staining experiments confirm that agrin is present at, and localized to, the synaptic basal lamina of neuromuscular junctions.

Proteoglycans

Proteoglycans are proteins that possess covalently attached carbohydrate chains known as glycosaminoglycans (GAGs). Proteoglycans are ubiquitous components of ECMs, and are also found on virtually all cell surfaces and within intracellular vesicles (including synaptic vesicles). From the point of view of their protein components (their "cores"), proteoglycans are certainly not a single family of molecules. Proteoglycans cores fall into many different families, and vary from very large (e.g., 400 kd) to very small (e.g., 10 kd); from membrane-spanning, to lipid-tailed, to secreted (for reviews see Gallagher, 1989; Lander, 1992).

In contrast, from the point of view of GAG structure, proteoglycans may be classified into three basic categories: those bearing GAGs of the heparinlike family, those bearing GAGs of the chondroitinlike family, and those bearing the GAG keratan sulfate. Each of these GAG types is synthesized onto the protein core as an unbranched linear polymer consisting of a simple disaccharide repeat; which of three basic disaccharide repeats is used determines which of the three families the GAG will belong to. Following chain polymerization, GAGs are chemically modified in a variety of ways. Most of these modifications introduce sulfate groups at various positions into some—but rarely all—of the disaccharide subunits. These sulfate groups give GAGs a strong negative charge. Another modification isomerizes glucuronic acid to iduronic acid.

One result of the chemical modifications that occur during GAG biosynthesis is to generate a great deal of complexity within each GAG family. It is likely that this is of considerable biological significance, since the biological functions of proteoglycans are likely to depend strongly on the binding properties of their GAG chains. Unfortunately, the full range of GAG complexity *in vivo* is not well understood, nor does any simple nomenclature exist that adequately conveys such complexity. Instead, GAGs are usually referred to by broad terms such as heparan sulfate (all members of the heparinlike family with the exception of heparin itself—a highly modified species produced only by mast cells); chondroitin sulfate (members of the chondroitin family containing little to no iduronic acid); dermatan sulfate (members of the chondroitin family containing large amounts of iduronic acid); and keratan sulfate. Although these labels are convenient for some purposes, those reading the literature should keep in mind that the "same" GAG chain—e.g., heparan sulfate—on any two proteoglycans may be structurally very different.

Perhaps in view of the inadequacies of GAG nomenclature, it has become standard practice to name proteoglycans according to their core proteins only. Thus, perlecan is a heparan sulfate proteoglycan found in basement membranes, while syndecan is a cell-surface proteoglycan that contains both heparan sulfate and chondroitin sulfate. One potential difficulty with this practice is the fact that, in a few cases, a core protein may bear one class of GAG in one cell type, a different type of GAG in another cell type, and no GAG at all in yet another.

Although heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate are all present in the nervous system (the first two being the most abundant), the considerable number of core proteins that bear these chains are only just now being identified. Many of these exhibit interesting developmental regulation of their expression (Herndon and

Lander, 1990). Some are integral membrane proteins, and are therefore not true components of the ECM. The remainder include some that are not readily extractable from neural tissue (as might be expected for an ECM component) and others that are surprisingly easy to solubilize. Immunohistochemical studies suggest that even these latter proteoglycans are frequently found in the ECM, suggesting that some of interactions holding proteoglycans in the nervous system ECM may be very weak.

Examples of proteoglycans that appear to be components of the ECM of the CNS are T1-PG, a widely distributed chondroitin sulfate proteoglycan (Iwata and Carlson, 1991a); 1D1, 3F8, and 3H1, two chondroitin sulfate proteoglycans and one mixed chondroitin- and keratan sulfate proteoglycan, respectively (Rauch et al., 1991); cytotactin-binding proteoglycan, a chondroitin sulfate proteoglycan that copurifies with tenascin (Hoffman et al., 1988); claustrin, a keratan sulfate proteoglycan (Cole and McCabe, 1991); and a chondroitin sulfate proteoglycan recognized by monoclonal antibody Cat-301 (Zaremba et al., 1989). The molecule recognized by Cat-301 is intriguing in that its expression surrounding neurons is controlled by neural (i.e., electrical) activity during development. Interestingly, no heparan sulfate proteoglycans have been identified as components of the ECM of the CNS. Whether it is only a matter of time before some are, or whether the CNS generally lacks heparan sulfate in the ECM, remains to be determined.

Proteoglycans are also found in the ECMs encountered by the PNS. In addition to many that are common components of interstitial matrices, some are associated with basement membranes of the PNS (e.g., Iwata and Carlson, 1991b), and one appears to be localized to the neuromuscular junction (Anderson and Fambrough, 1983).

Hyaluronic Acid- and Hyaluronate-Binding Proteins

Whereas most types of glycosaminoglycans are found as side chains of proteoglycans, one glycosaminoglycan, hyaluronic acid or hyaluronan, is not found covalently attached to protein. Hyaluronic acid is the simplest of GAGs, consisting of a linear polymer of the disaccharide [D-glucuronic acid $\beta(1\rightarrow3)$ D-N-acetylglucosamine $\beta(1\rightarrow4)$] that is not further chemically modified (e.g., by sulfation) as are other GAGs. Although structurally simple, hyaluronic acid chains are very long (M_rs in the range of 1,000 kd are common), giving hyaluronic acid solutions some unique biophysical properties. Among these properties is the ability to bind large amounts of water, and hyaluronic acid has been suggested to play widespread roles in controlling tissue hydration and the sizes of cell-free spaces *in vivo*.

The developing brain is particularly rich in hyaluronic acid. In addition, several brain ECM proteins have been identified to be hyaluronic acid-binding proteins. These include brain-specific hyaluronectin, a 68 kd protein, and glial hyaluronate-binding protein, a 60 kd protein (Perides et al., 1989). Glial hyaluronate-binding protein appears to occur only in the white matter of brain and spinal cord. Its amino acid sequence suggests that it may be derived (by alternative splicing or proteolytic cleavage) from the hyaluronic acid-binding domain of the core protein of the proteoglycan versican.

Collagens

Collagens are a large family of ECM glycoproteins. Collagen molecules consist of homo- or heterotrimers of polypeptides that contain regions of "collagenous" structure (amino acid sequences in which every third residue is glycine, and high levels of proline, lysine, hydroxyproline, and hydroxylysine are found). Because the virtual absence of collagens is a hallmark of the ECM of the CNS, collagens have not been extensively studied by neurobiologists. They are, however, abundant components of the ECM encountered by peripheral neurons and axons. Type IV collagen, in particular, is an abundant component of basement membranes, including those contacted by peripheral nerves during development and regeneration.

Other ECM Molecules

In recent years, several ECM molecules of unknown function have been identified in the nervous system. One of these, P30, is a small heparin-binding protein found at high levels in the developing CNS and PNS (Daston and Ratner, 1991). Another, SC1, was isolated by expression cloning from a rat brain cDNA library using an antibody directed against synaptic junction proteins. The cDNA cloned encodes a protein with a predicted size of 71 kd (the apparent size of the molecule synthesized *in vivo* is 120 kd) that exhibits substantial similarity to the ECM glycoprotein known as osteonectin or SPARC (Johnston et al., 1990). SC1 appears to be expressed by many cells of the adult CNS, including neurons of the cerebellum, cerebral cortex, and hippocampus. Most recently, a molecule showing some similarity to thrombospondin has been cloned and localized to the floor plate of the developing spinal cord (Klar et al., 1992). This protein is predicted to be 91 kd in size, and is also detected in adult kidney and brain.

Matrix-Associated Molecules

Although the above discussion has focused on what might be termed the "structural" components of ECM—those molecules that are abundant enough to play a role in determining overall ECM structure—there are many other molecules that, while normally thought of as soluble pro-

teins, probably spend much of their time *in vivo* physically bound to the ECM. These molecules include polypeptide growth factors, secreted proteases and protease inhibitors, and other extracellular enzymes. Examples of each type of molecule are found in the nervous system: These include the acidic and basic fibroblast growth factors, the protease thrombin, the protease inhibitor known as protease nexin-1 (also known as glial-derived nexin), and one form of the synaptic enzyme acetylcholinesterase (Brandan et al., 1985; Dihanich et al., 1991; Eckenstein et al., 1991; Gloor et al., 1986). In all cases, it is interaction with glycosaminoglycans, particularly heparan sulfate, that appears to bind these molecules to the ECM. The biological significance of matrix association for these molecules is unclear. Potentially, the ECM can act to precisely localize these molecules (as in a synapse) or act as a storage depot, permitting the molecules to be mobilized by local ECM degradation. Alternatively, the ECM may alter the intrinsic properties of these molecules. Heparin and heparan sulfate are known to have such an effect on many proteases and on protease nexin-1, and appear to play a critical role in the functioning of acidic and basic fibroblast growth factor as well. Finally, association with the ECM is thought to dramatically influence the sensitivity of these molecules to proteolytic degradation. Clearly, much remains to be learned about matrix-associated molecules in the nervous system.

AMYLOID: A PATHOLOGICAL ECM

Under the broadest definition of ECM—insoluble material found between cells—must be included those extracellular deposits that occur in certain disease states. The amyloids are a large group of such deposits, and they are charactertized by a relatively uniform histological appearance, even though their molecular composition may be quite different depending on the disease process involved. Amyloidosis involving the nervous system is a key feature of Alzheimer's disease, as well as the so-called prion encephalopathies (Creutzfeld-Jakob disease, Gerstmann-Straussler syndrome, kuru).

As it turns out, the similarities between amyloids and normal ECMs go beyond the mere fact that both are insoluble. Mounting evidence indicates that proteoglycans are common, possibly invariant, components of amyloid deposits—including those in Alzheimer's and prion diseases—and suggests that proteoglycans may be involved in the initial formation of amyloid plaques (see Snow and Wight, 1989, for review). Other typical ECM components have also been identified in amyloid deposits, as have molecules such as protease inhibitors, which tend to associate with normal ECMs as well. These findings suggest that amyloid deposits may exhibit a structural organization not unlike that of conventional ECMs.

BINDING INTERACTIONS AMONG MOLECULES OF THE NERVOUS SYSTEM ECM

Although the ECM of the brain lacks the mechanical strength of connective tissue matrices, it is nonetheless held together by a complex network of intermolecular interactions. Figure 2 summarizes what is currently known about the binding interactions that occur among the ECM molecules described above. Taking for granted that many more interactions are likely still to be discovered, it is apparent that the ECM of the nervous system—like ECMs elsewhere—is potentially very complex in its organization. One interesting point that emerges from Figure 3 is the suggestion that proteoglycans may play a pivotal role in matrix organization in the nervous system, since the vast majority of ECM molecules interact directly with them. This interaction stems from the ability of most ECM proteins to bind heparan sulfate or, in a few cases, chondroitin sulfate. Just how important a role proteoglycans play in matrix organization will only become clear after the proteoglycan composition of the nervous system ECM is better understood.

BIOLOGICAL EFFECTS OF ECM

Molecules of the ECM have been shown to regulate cellular behaviors as diverse as proliferation, differentiation, migration, and, of particular interest in the nervous system, axon growth and guidance. In many cases, it has been possible to relate particular biological activities to particular domains of ECM molecules. Some of these relationships are illustrated in Figure 3. Such associations are based on experiments in which domain-specific antibodies, peptides derived from defined regions of amino acid sequence, and proteolytic fragments of ECM molecules, are used as reagents to mimic or block biological effects. One principle

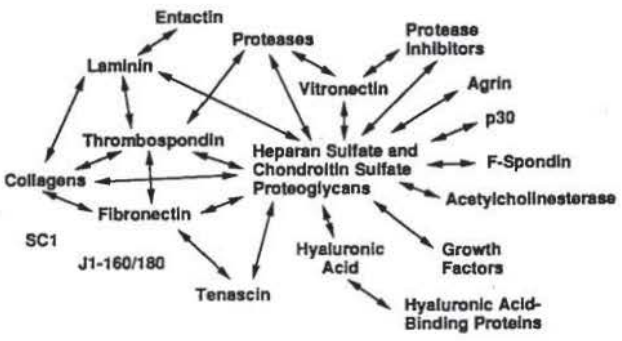

Fig. 2. Interactions among molecules of the ECM. Binding interactions that can occur between ECM molecules are indicated by arrows. Not indicated are examples of self-binding (aggregation or polymerization), which has been observed for laminin, fibronectin, thrombospondin, and collagens.

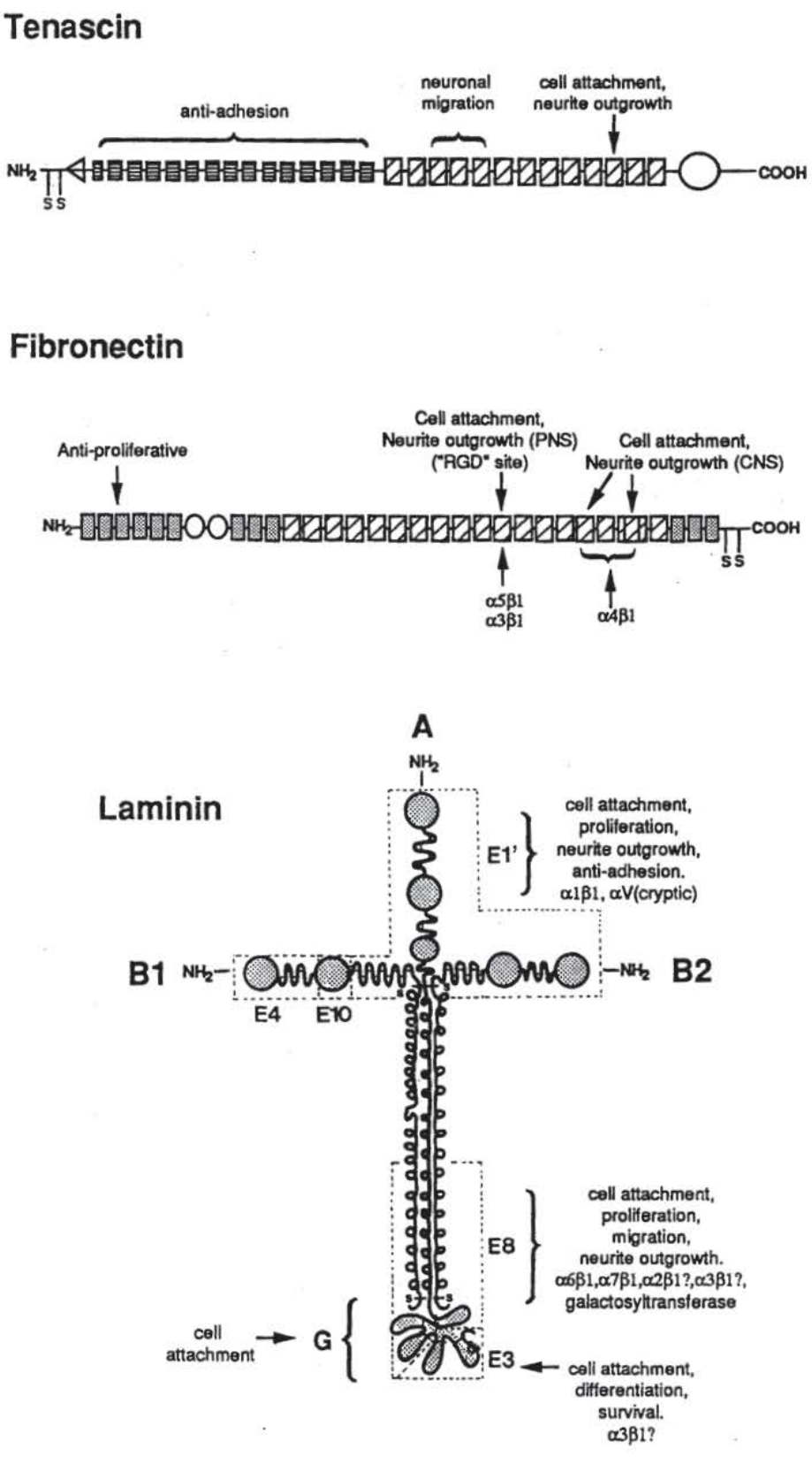

Fig. 3. Functional domains of ECM molecules. The association of biological activities and known receptor binding sites with particular domains of laminin, fibronectin, and tenascin are shown. For laminin, all three chains are named (A, B1, B2), and the regions comprised by elastase fragments (E1', E3, E8, E4, and E10) are indicated. Domain G of laminin represents the five shaded loops at the carboxyl end of the A chain, and partially overlaps the E3 and E8 fragments. For tenascin and fibronectin, only one of the polypeptide chains in each molecule is depicted. The diagram of laminin is modified from Yurchenco and Schittny (1990).

that seems to be emerging from such studies is that molecules of the ECM typically contain a number of spatially separate functional domains, and that these domains can have very different, even antagonistic, biological effects on cells. In this section, ECM effects on cells—especially on cells of the nervous system—are discussed as much as possible in terms of the molecules and molecular domains that elicit the effects. In the following section, the cellular receptors that mediate biological responses to ECM molecules are discussed.

Effects of ECM on Cell Proliferation, Differentiation, and Survival

Effects on cell proliferation, differentiation, and survival have been noted for laminin, fibronectin, thrombospondin, and tenascin. The suggestion that laminin may play a role in regulating cell proliferation has been made in several reports. In these studies, addition of soluble laminin to cell cultures, or growth of certain types of cells on laminin substrata, was shown to result in an increase in ^{3}H-thymidine incorporation and/or cell number, suggesting that laminin may have a mitogenic effect on some cells. There is as yet no consensus on the molecular domain(s) that mediate laminin's putative mitogenic effect, and there may be more than one: A peptide from the laminin A chain that contains the amino acid sequence IKVAV and is found in the E8 proteolytic fragment of laminin appears to be mitogenic for PC12 pheochromocytoma cells (Kubota et al., 1992), whereas a proteolytic fragment (E1-4) containing regions from the amino-termini of all three chains (A, B1, B2) stimulates growth of 3T3 fibroblasts (Panayotou et al., 1989)(see Fig. 3 for structures of laminin fragments). Laminin has also been shown to stimulate neuronal differentiation (i.e., the expression by cells of proteins that are characteristic of a neuronal phenotype) in adrenal chromaffin cells, a neural crest-derived cell type that possesses some biochemical characteristics of catecholaminergic neurons (Acheson et al., 1986). In this case, the biological effect has not been completely mapped, but sequences in the E3 fragment (part of the G-domain; see Fig. 3) are necessary. A similar requirement for the E3 domain has been noted for laminin's ability to promote neuronal survival under conditions where trophic factor support is limiting or absent (e.g., Edgar et al., 1984).

Fibronectin, in contrast to laminin, has been shown to have a growth inhibitory effect on some cells of the nervous system. This activity of fibronectin appears to be "cryptic", i.e., a proteolytic fragment of fibronectin, but not the intact molecule, possesses it. Growth control of normal Schwann cells by fibronectin appears to be part of an autocrine feedback loop, in which mitogen-stimulated cells secrete a metalloprotease that generates an amino-terminal fragment of the fibronectin molecule; it is this fragment (the 29 kd amino-terminal, heparin-binding fragment of fibronectin) that inhibits Schwann cell proliferation (Muir and Manthorpe, 1992). Interestingly, transformed Schwann cells do not secrete the activated form of the metalloprotease, suggesting one mechanism by which these cells might escape regulated growth control. A different domain of the fibronectin molecule appears to mediate effects on cell differentiation. This has been studied most extensively in cultures of human epidermal keratinocytes, where fibronectin has been shown to block gene expression characteristic of differentiated cells. This effect is mediated by the cell-binding fragment of fibronectin, and by a peptide (GRGDSP) contained within this fragment that is known to bind cellular ECM receptors of the integrin class (Adams and Watt, 1989). Fibronectin may be able to modulate expression of differentiated characteristics by neural crest cells as well (cf. Rogers et al., 1989).

Although both thrombospondin and tenascin have been shown to have mitogenic effects on cells (e.g., Chiquet-Ehrismann et al., 1986; Majack et al., 1986), there are as yet no reports of such effects on cells of the nervous system. It has been suggested that thrombospondin may function as part of an autocrine growth control pathway for vascular smooth muscle cells (Majack et al., 1986). A possible function for thrombospondin as an autocrine growth-stimulating factor would be particularly interesting in the developing nervous system, since widespread expression of thrombospondin has been observed at a time (E13 in the mouse embryo) when neuronal proliferation is proceeding at a high rate (cf. O'Shea and Dixit, 1988).

Effects of ECM on Axon Growth and Guidance

Laminin is well known as a potent stimulator of process (neurite) outgrowth by many types of cultured neurons, and its presence *in vivo* along pathways that growing axons follow (described above) supports the notion that it plays an important role in promoting axon growth during nervous system development (reviewed in Reichardt and Tomaselli, 1991; Sanes, 1989). *In vitro*, laminin is capable not only of stimulating neurite growth but also of precisely guiding it. Growth cones, the growing tips of neurites, accurately follow substratum pathways along which laminin has been deposited (cf. Gundersen, 1987). The neurite outgrowth effects of laminin appear to involve different molecular domains, depending on the cell type tested. For many neurons, this activity apparently resides in the E8 proteolytic fragment; however, for some the major neurite outgrowth-promoting activity maps to the E1-4 region (Fig. 3; reviewed by Beck et al., 1990).

Fibronectin's role in mediating axon growth and guidance during nervous system development is less well understood than that of laminin. Those CNS neurons that have been tested so far do not readily extend neurites on fibronectin substrata, but many PNS neurons do. Oddly, some CNS neurons (embryonic spinal cord) do extend neurites on a 33

kd proteolytic fragment of fibronectin, implying that this region of the molecule contains "cryptic" neurite outgrowth-promoting activity (Rogers et al., 1989). Unfortunately, much of the analysis of the effects of fibronectin on CNS neurons preceded studies of fibronectin expression in the brain (described above). In view of fibronectin's expression in the subplate of the cerebral cortex (Stewart and Pearlman, 1987), it would seem particularly valuable to test fibronectin for effects on those neurons that send axons into that region, namely thalamic and cortical projection neurons.

Tenascin, thrombospondin, and vitronectin, like laminin and fibronectin, have all recently been shown to stimulate neurite outgrowth by some classes of neurons (reviewed by Tomaselli and Neugebauer, 1991). The fact that so many ECM molecules can promote neurite outgrowth has raised the possibility that mere cell attachment to an ECM molecule might be all that is required for neurite extension. While this might be true for a few types of neurons, it does not appear to be the case generally. Indeed, degree of cell–substratum adhesion and degree of stimulation or guidance of neurite growth by ECM molecules do not correlate well (Gundersen, 1987; see Lander, 1990, for discussion). In fact, some neurons readily extend neurites in response to ECM molecules to which the cells themselves don't attach appreciably (Calof and Lander, 1991; Wehrle and Chiquet, 1990). Moreover, for some neurons, molecules that stimulate neurite outgrowth actually antagonize cell–substratum adhesion. This property has been termed anti-adhesiveness, and has been observed with neurons interacting with both laminin (Calof and Lander, 1991) and tenascin. Laminin's anti-adhesive activity appears to map to the E1' region of the molecule (Fig. 3; Calof et al., 1992). Tenascin's separable biological activities include not only neurite outgrowth-promoting activity (which maps to a site within its fibronectin-type III repeat units) but cell attachment activity (which maps to a potentially different site in the fibronectin type III repeats), anti-adhesive activity (which maps to the epidermal growth factor-like repeats near the amino-terminus), and a "neurite-repulsive" activity, i.e., the ability to render a substratum nonpermissive for neurite growth that has not yet been mapped (Fig. 3; Lochter et al., 1991; Spring et al., 1989). Interestingly, the ability to be either adhesive or "repulsive" (for cells or neurites) is also a characteristic of the tenascin-related molecule J1-160/180 (Morganti et al., 1990).

The neurite outgrowth-promoting activities of thrombospondin and vitronectin have not yet been mapped to specific domains, although there has been one report that thrombospondin's activity can be found in a 140 kd fragment containing the carboxy-terminal and cell-binding domains (O'Shea et al., 1991).

Understanding the role(s) that proteoglycans play in modulating axon outgrowth and guidance is made difficult by the tremendous complexity of this class of molecules and the large number of them expressed in the nervous system (cf. Herndon and Lander, 1990). No unifying concepts have yet emerged, and it is probably naive to expect proteoglycans all to exert similar effects on neurons. Several groups have reported that chondroitin sulfate and keratan sulfate proteoglycans inhibit neuronal attachment and neurite outgrowth, and in this light it has been suggested that proteoglycans may function in forming barriers to axon outgrowth during nervous system development (e.g., Cole and McCabe, 1991). At least one report, however, has suggested that the core protein of a brain-derived proteoglycan may stimulate neurite outgrowth by cortical neurons (Iijima et al., 1991).

Effects of ECM on Cell Migration

Neural crest cells, the mesenchymal cell type that gives rise to the peripheral and autonomic nervous systems, constitute the neural cell type whose migratory response to ECM has been studied most extensively. Fibronectin, collagens, laminin, and tenascin—all of which can be found in areas where neural crest cells migrate *in vivo*—all support neural crest cell migration in culture to some extent (e.g., Halfter et al., 1989; Perris and Bronner-Fraser, 1989; Reichardt and Tomaselli, 1991). It is possible that neural crest cells are intrinsically highly motile, and will migrate on any substratum that supports adequate attachment. The pathways they follow *in vivo* might then be determined more by the locations of molecules that inhibit their migration than by those that stimulate it. Some studies suggest that proteoglycans play such a migration-inhibiting role for these cells (cf. Perris et al., 1991).

In contrast to the behavior of neural crest cells, the migratory responses of other neural cells suggest that molecules of the ECM can function in an instructive manner, to specifically stimulate and guide cell migration. An interesting example of this specificity is the case of neurons and neuronal precursors of the nasal olfactory epithelium. *In vivo*, neuronal cells derived from the developing nasal epithelium migrate along nerve fiber tracts into the brain, where they give rise to the luteinizing hormone-releasing neurons of the hypothalamus. *In vitro*, neuronal cells from the embryonic olfactory epithelium are highly motile on substrata coated with laminin or its A-chain isoform, merosin, and migration of these cells is stimulated and guided by laminin but not by fibronectin, collagens, or nonspecifically adhesive substrata such as polylysine. Recent work suggests that laminin's effect on migration of these cells maps to the E8 fragment of the molecule (Fig. 3; Calof et al., 1992). It is interesting that the migration-stimulating activity of laminin maps neither to the molecule's anti-adhesive site (see above) nor to its most adhesive site for these cells, which is found in the G-domain, and involves the E3 region (Calof et al., 1992).

Migration of granule cells during histogenesis of the cerebellum may prove to be an example of ECM molecules acting sequentially to regulate neuronal migration through a complex and changing cellular environment (reviewed in Reichardt and Tomaselli, 1991). Thrombospondin appears to play an important role in facilitating migration of granule cells from the external granule layer (EGL) into the molecular layer of the developing cerebellum, since antibodies to thrombospondin prevent granule cells from entering the molecular layer in cultured cerebellar slices. Tenascin apparently functions at the later stage of promoting granule cell migration through the molecular layer: Antibodies to tenascin cause granule cells to accumulate in the molecular layer, failing to complete their migration through it and into the internal granule layer. A recent study utilizing domain-specific, function-blocking monoclonal antibodies in cerebellar slice cultures indicates that the migration-promoting activity of tenascin maps to a domain between the third and fifth fibronectin type III repeats in the carboxy-terminal portion of the molecule (Fig. 3; Husmann et al., 1992). This domain of tenascin is close to, but separate from, the domain that contains neurite outgrowth-promoting, anti-adhesive, and attachment activity.

CELLULAR RECEPTORS FOR ECM

Studies of the mechanisms of cell response to the ECM have concentrated in recent years on the search for cellular receptors for ECM molecules. Several of the classes of cell-surface molecules that have the ability to bind ECM components are described below, and are summarized in Table 1.

Structure and Function of the Integrin Family of Receptors

The most widely studied class of cellular receptors for ECM molecules is the superfamily of integral membrane proteins known as integrins. A recent review describes the structure and function of the members of this large (20 known members and still growing) and complex group of receptors, and the reader is referred to that paper for a detailed discussion of integrins (Hynes, 1992). For the purposes of this discussion, some basic features of integrin structure are summarized.

The integrins are heterodimers of noncovalently linked subunits, referred to as α and β Both subunits are transmembrane glycoproteins, and both appear to contribute to ligand binding by the dimer. The structural and functional complexity of the integrins appears to be the result of two factors: formation of different heterodimers, resulting from different pairings of the eight β subunits and the 13 α subunits, and structural variation in the subunits themselves, resulting from alternative splicing of their cytoplasmic domains.

TABLE 1. Known Interactions of ECM Proteins with Cell Surface Molecules

	Integrins	Proteo-glycans	Glyco-lipids	Others
Laminin	+	+	+	Lectins, galacto-syltransferase, other proteins
Fibronectin	+	+	+	Other proteins
Thrombo-spondin	+	+	+	
Tenascin	+	+		
Vitronectin	+	+		
Collagens	+	+		Other proteins
Hyaluronic acid		+		CD44/H-CAM

Integrins, the majority of which appear to function as receptors for ECM molecules (but see Hynes, 1992, and Hynes and Lander, 1992), are expressed by virtually every cell type, and most cells express more than one integrin. So far as is known, each of the 20 or so heterodimers has a distinct ligand specificity. However, it is important to point out that this does not mean that each integrin heterodimer can bind only one ligand. Many integrins can bind more than one ECM molecule, and a given ECM molecule can be recognized by more than one integrin. For example, there is evidence for at least six integrin "laminin receptors"—α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4—and the first three of these are known to function as receptors for other ECM molecules as well (cf. Hynes, 1992).

Integrin receptors appear to be important in mediating attachment, migration, and axon outgrowth by neural cells in response to ECM. The avian retinotectal pathway provides an example of how regulation of integrin expression and function may be important in governing axon outgrowth during nervous system development: At a time in development when retinal ganglion cells are sending axons along a laminin-rich pathway to the brain (embryonic day 6 in the chick), they are highly responsive to laminin, as shown by neurite outgrowth assays on laminin substrata *in vitro*. Later in development, at the time when most retinal ganglion cell axons have reached their targets in the laminin-poor optic tectum (around embryonic day 11 or 12), the ability of retinal ganglion cells to extend neurites on laminin substrata is lost (Cohen et al., 1986). The time course of loss of responsiveness to laminin correlates with decreased expression of the α6 integrin subunit by retinal ganglion cells, suggesting that regulation of integrin laminin receptor (α6β1) expression is one means by which neuronal responsiveness to the ECM is regulated (de Curtis et al., 1991). Interestingly, other retinal neurons also lose the ability to attach to and extend neurites on laminin at embryonic day 11–12, but α6 expression is not down-regulated in these cells. This suggests that an integrin's

function may be suppressed, even when it is present at normal levels on the cell surface, and that this may be a second means by which neuronal responsiveness to the ECM is regulated during development. This idea is supported indirectly by the existence of a monoclonal antibody that binds the β1 integrin subunit and effectively restores the ability of embryonic day 11 retinal neurons to respond to laminin (Neugebauer and Reichardt, 1991). Since this antibody recognizes a site on the β1 integrin subunit that is not involved in ligand binding, its activity appears to represent a case of allosteric modulation of integrin function. In other words, it appears that integrins can exist in active and inactive conformations that can be interconverted through the action of other integrin-binding molecules. Evidence suggests that signals inside cells, as well as molecules outside cells, can control integrin activation (Hynes, 1992).

Although multiple integrins can act as receptors for the same ECM protein, the locations of binding sites for integrins on proteins tend to vary (Fig. 3). The locations of integrin binding sites are in turn directly related to the biological activities of some of the molecular domains that were discussed in the previous section. For example, the neurite outgrowth-promoting activity displayed by the E1' fragment of laminin requires the receptor function of integrin α1β1, which binds to E1', while the neurite outgrowth-promoting activity of the E8 fragment requires either integrin α3β1 or α6β1, which interact with sites in the E8 fragment (Tomaselli et al., 1990). It is likely that differences in the responses of different cell types to an ECM molecule and its fragments frequently result from differences in integrin expression.

Clearly, it will be important to characterize the expression of integrins by many of the cell types in the nervous system, a process that has only just begun. In theory, one might be able to predict some of the responses of a cell to its ECM simply by knowing its complement of integrins and the ECM molecules it is encountering. Unfortunately, things are unlikely to be that simple. Not only are integrins sometimes present but inactive on cell surfaces (as described above for some retinal neurons), but a single type of integrin may mediate different biological responses in different cells. For example, the integrin α6β1 mediates cell adhesion to laminin in many cells, neurite outgrowth in response to laminin in other cells (e.g., retinal cells), and stimulation of migration without stimulation of cell adhesion in other cells (e.g., olfactory receptor neurons; Calof et al., 1992).

These considerations suggest that the key to understanding how integrins mediate cellular responses to the ECM will lie in understanding the intracellular signals transduced by integrins, and how these signals converge with others to affect cell behavior. The idea that integrins transmit signals into cells has only recently gained wide acceptance, as evidence accumulates that integrin interactions with ECM molecules can elicit some of the hallmarks of signal transduction (e.g., protein tyrosine phosphorylation, elevation of cytoplasmic pH). For a recent discussion of this emerging field, the reader is referred to the review by Hynes (1992).

Nonintegrin Receptors for ECM Molecules

Several other cell-surface proteins, besides integrins, may function as receptors for ECM. A 100 kd protein isolated from brain on the basis of its ability to bind laminin is one of several candidate nonintegrin laminin receptors (Smallheiser and Schwartz, 1987). Two others may function as lectins: CBP35 (a 35 kd carbohydrate-binding protein also known as Mac 2), a laminin-binding protein expressed on macrophage cell surfaces (Woo et al., 1990), and a 67 kd laminin-binding protein isolated from tumor cells, which may be identical to an elastin receptor that functions as a galactose-specific lectin (Mecham et al., 1989; Sephel et al., 1989). Functional roles for these carbohydrate-binding proteins in mediating biological activities of laminin such as adhesion, migration, and neurite outgrowth have not yet been established. However, there is evidence that the carbohydrate moieties present on laminin do play a role in these activities, since neurite outgrowth by PC12 pheochromocytoma cells is impaired on unglycosylated laminin (reviewed by Mercurio, 1990).

Evidence from investigations of another putative laminin receptor also indicates that carbohydrate groups on laminin are important to neural cells. Cell-surface β(1→4)-galactosyltransferase is a cell-surface enzyme that appears to play some role in several cell interactions with laminin, including cell migration and neurite outgrowth. Galactosyltransferase binds to N-linked oligosaccharides on laminin, and interaction of this enzyme with oligosaccharides present on the E8 region of the molecule is apparently important for PC12 pheochromocytoma cell neurite outgrowth on laminin (Begovac et al., 1991). Neural crest cells also express galactosyltransferase, and agents that interfere with the activity of the enzyme, or antibodies directed against it, perturb neural crest migration *in vivo* and *in vitro* (Hathaway and Shur, 1992).

As described earlier, most ECM proteins possess domains that bind to the GAGs found on proteoglycans. Since proteoglycans are found not only in the ECM but also on most cell surfaces, it is not surprising that proteoglycans have been suggested to act as receptors for ECM molecules. The best studied proteoglycan in this regard is syndecan, a transmembrane protein that bears both heparan sulfate and chondroitin sulfate chains. Syndecan can mediate adhesion of epithelial cells to fibronectin, collagen, and thrombospondin (Bernfield and Sanderson, 1990; Sun et al., 1989). Although syndecan is not detectable in the CNS, other integral membrane proteoglycans are, including N-syndecan (a proteoglycan with a region of homology to syndecan; Carey et al., 1992), NG2 (a proteoglycan present on certain glial cells; Nishiyama et al., 1991), glypican (Litwack et al.,

1992), and several molecules that have been only partly characterized (Herndon and Lander, 1990). Interestingly, some of these proteoglycans are linked to the plasma membrane via phosphatidylinositol glycan linkages, providing a potential mechanism by which proteoglycans might function to transmit signals into cells.

Finally, several ECM molecules have been found to bind various types of cell-surface glycolipids, especially gangliosides and sulfated lipids (Table 1). It is not yet known whether such molecules act as true receptors for ECM molecules. However, if such a role should be demonstrated, it will most likely have important implications for the nervous system, since a high abundance and diversity of glycolipid expression is a characteristic of neural tissue.

CONCLUSIONS

In only a few decades, the ECM of the nervous system has progressed from being an entity not widely recognized to exist to being a critical controller of virtually all aspects of cellular behavior. The functions of the nervous system ECM derive not merely from its ability to act as a scaffold or a glue to bind cells together. Instead, the ECM consists of numerous biologically active components, each containing distinct functional domains capable of having a wide variety of effects on cells. The effects of ECM molecules are mediated through diverse cellular receptors, including integrins, proteoglycans, lectins, cell-surface enzymes, and other molecules. One challenge of the next few years will be to determine how the signals transmitted to cells via the ECM cooperate with developmental signals to drive the morphogenesis of the nervous system.

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