Extracellular matrix and neuronal movement

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Summary. During brain development, both neuronal migration and axon guidance are influenced by extracellular matrix molecules present in the environment of the migrating neuronal cell bodies and nerve fibers. Glial laminin is an extracellular matrix protein which these early brain cells preferentially attach to. Extracellular glycosaminoglycans are suggested to function in restricting neuronal cell bodies and axons from certain brain areas. Since laminin is deposited along the radial glial fibers and along the developing nerve pathways in punctate form, the punctate assemblies may be one of the key factors in routing the developing neurons in vivo. This review discusses the role of laminin in neuronal movement given the present concept of the extracellular matrix molecules and their proposed interactions.

Key words. Axon guidance; brain development; extracellular matrix; laminin; neuronal migration.

Introduction

Extracellular connective tissue components, in particular laminin, play an important role in early development and mature functioning of several organs[54]. Since the beginning of the 1980's, many of these proteins or their isoforms have been localized in nervous tissue[44] where they have been suggested to play a role in the cell-to-cell interactions upon which neuronal migration and nerve pathway formation are based. Several up-dated reviews on the characteristics and location of matrix proteins[4,5,25,26,68,85], their receptors[39,83] and extracellular matrix in the nervous system[71] satisfactorily cover the earlier work and the milestones of research in these fields. This review focusses on the role of connective tissue molecules in early brain development, particularly on the role laminin(s) play in facilitating neuronal movement.

Extracellular matrix proteins in the embryonic brain

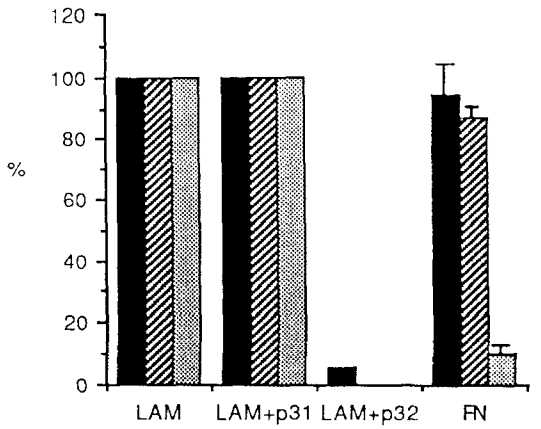
Of the known extracellular matrix proteins, laminin, fibronectin[71], thrombospondin[59], tenascin[36] and glycosaminoglycans[1,28,75] (GAGs) are all present in embryonic brain during the period of neuronal migration and axon pathway formation. Laminin is localized along the routes of migrating neurons and axons[71], which implies that glial laminin may be a substratum central neurons use for their attachment and neurite extension. The cellular origin of fibronectin in embryonic brain is still controversial. Its presence on the surface of subplate neurons[13,78] suggests that fibronectin from the cerebrospinal fluid or plasma may bind to these cells. When glial cells are allowed to age in culture they will synthesize a fibronectin isoform[48,63] that differs from fibroblast fibronectin by alternative splicing of the message[72].

It is possible that cell surface associated fibronectin regulates neuronal migration. Binding of fibronectin to the cellular receptors, possibly common for laminin and fibronectin[39], may prevent developing neurons that migrate through the subplate from recognizing laminin or related guidance cues. A modulatory role for fibronectin for central neurons is supported by our recent observations: we have shown that fibronectin induces neuronal attachment and neurite outgrowth of central neurons when used as substratum for neurons cultured without serum (fig. 1). This effect of fibronectin is apparently masked by serum components[45]. However, this fibronectin effect is less dramatic than that of laminin: fibronectin does not promote outgrowth of long neurites, which is the most characteristic effect of laminin on central neurons (fig. 2).

Immunocytochemical studies locate thrombospondin[3,59] and tenascin[31,35,42] (J1 or cytotactin) to embryonic brain tissue. Both of these matrix proteins bind proteoglycans: thrombospondin binds to heparansulfate proteoglycans[26] and tenascin to chondroitin sulfate[25]. Binding of tenascin to fibronectin has been observed with the chicken protein[11,27], but not with the mammalian protein[25]. Thrombospondin further binds to laminin, fibronectin, and collagen types I and V[26]. Future studies need to address the question of whether these matrix proteins will influence neuronal migration or axon guidance.

Current studies indicate that tenascin is a poor adhesive substrate for most cells tested[25]. For example, chicken neurons extend neurites on tenascin, but slowly and only after the neuronal attachment has been stimulated by other adhesives such as poly-L-lysine (Chiquet-Ehrismann, personal communication). In fact, the location of tenascin along neural crest cell pathways[33,53,77] suggests that it may be one of the modulatory inhibitors of cell movement. This may be partially related to its binding affinity to chondroitin sulfate – another putative neuronal repulsion substance[75,76].

Laminin is found along the pathways of early neuronal migration[46] and along the routes taken by pioneering axons[71]. In contrast, keratan sulfates are found in places that axons avoid in vivo[75], and in vitro studies indicate that the local concentration gradients of keratan and chondroitin sulfates and laminin may have differential control of neuronal migration and axonal growth[76]. In



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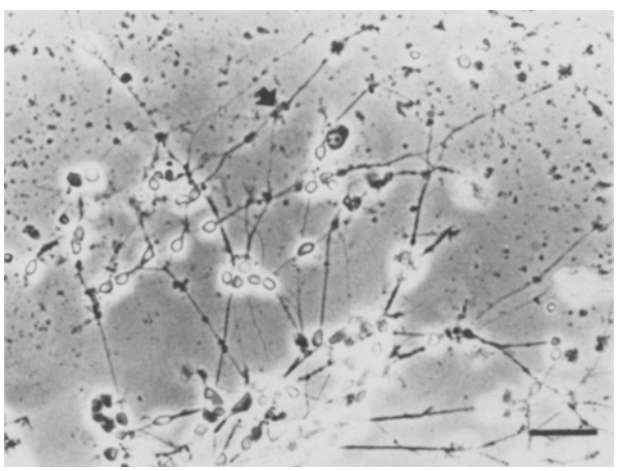

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Figure 1. The effect of fibronectin substratum on neurite outgrowth of rat cerebellar neurons grown in the absence of serum. in *(a)*, neuronal attachment (■), and outgrowth of short (▨) and long (⊡) neurites was evaluated on laminin (LAM) and fibronectin (FN) substratum. On laminin effects of high concentrations of different synthetic peptides added into the culture medium was also investigated (see Liesi et al.[52] for peptide information). As compared to laminin, fibronectin was as good in neuronal attachment and outgrowth of short neurites. No long neurites were seen on fibronection substratum. Results from six separate experiments are expressed as a percentage (means ± SEM) of the neuronal attachment and neurite outgrowth as compared to the laminin substrate. In *(b)* neurons with short neurites spread on fibronectin substratum.

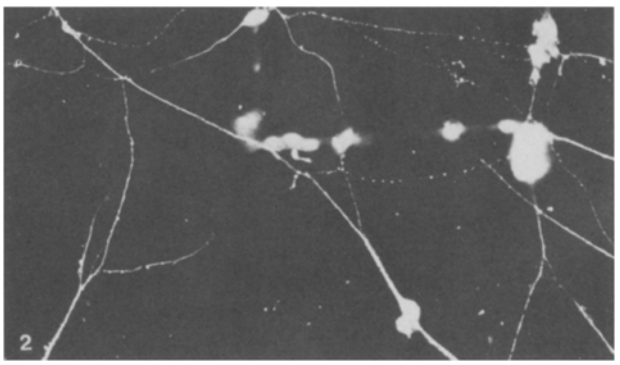

Figure 2. Neurofilament staining of rat embryonic cortical neurons grown on the basement membrane laminin substratum. Laminin induces extensive neurite outgrowth of long neurites in less than 12 h.

other words, the growth inhibition by these GAGs can be abolished by increasing the local laminin concentration and vice versa.

Interestingly, heparansulfate proteoglycans often complex with laminin under conditions that support neuronal migration and neurite outgrowth[12,18]. Since all glycosaminoglycans tested have been shown to support little or no neurite outgrowth in vitro[8] the presence of heparansulfate proteoglycans (HSPGs) may organize specific molecular interactions more favorable for neurite outgrowth. For example, many growth factors (e.g. fibroblast growth factor) bind heparin[84] and so do most of the matrix proteins, such as laminin, fibronectin and thrombospondin. By binding these molecules HSPGs may augment molecular interactions necessary for neuronal movement and axonal growth.

It has been suggested that neurons do not migrate along a concentration gradient of laminin in vitro[56]. No concentration gradient of laminin along radial glial fibers or along axon pathways has been demonstrated in vivo[15,32,46,49]. This implies that the punctate deposits of laminin may provide the stepping stones for neurons and axons, but are not giving them directionality. Soluble, target-released yet unidentified factors have been shown to be able to direct axons[34,35,80]. Whether such soluble factors may also direct neuronal migration is not known.

Since the appearance of the punctate form of laminin precedes the growth of pioneering axons[49] and correlates with neuronal migration[46], it is possible that migrating neurons and pioneering axons may release factors that induce glial cells in their vicinity to deposit laminin in the extracellular membrane bound form. These extracellular punctate deposits of laminin would then be utilized in neuronal migration and neurite extension. Laminin synthesis by glial cells could further be down-regulated by the contact of migrating cell bodies and nerve fibers with the extracellular punctate deposits of laminin. This might provide a simple and efficient way to prevent latter neurons and axons from following the same route. Since there are obviously situations where axons or nerve cell bodies must make choices between different routes that all have extracellular punctate deposits of laminin, other molecules such as glycosaminoglycans, fibronectin or additional factors could play a regulatory role.

This hypothesis implies that punctate deposits of laminin may be needed for the initial steps of migration and axon growth. The various cell adhesion molecules[69] (CAMs) and growth factors[84] may then co-operate and play a role in stabilizing cell-to-cell interactions. On line with the requirement for a second wave of molecules, is the observation that retinal ganglion cells do not survive on laminin past a certain time in culture although they will

survive on glial cells [15]. The glial cells may produce additional molecules needed for long-term survival of neurons in culture [24, 66].

Basement membrane laminin and neuronal development

Because of its availability, the EHS-tumor laminin is the most widely used to study neurite outgrowth, and most laminin antibodies available are raised against this tumor cell derived protein. This laminin is a potent promotor of neurite outgrowth in vitro [71]. However, the glial laminin differs considerably from this basement membrane analogue [50]. Our studies suggest that glial laminin may have entirely different structural properties (fig. 3) and, hence, different molecular interactions. Molecular differences between isoforms of laminin may explain why neurite outgrowth on glial cells is not inhibited by antibodies to the EHS-tumor derived laminin [16], although such antibodies can inhibit neurite outgrowth on purified laminin [20, 45].

It is known that the most antigenic site of laminin is the P1 fragment of the molecule [81], which is located in the short arms of the protein far away from the neurite outgrowth promoting region [21, 51]. Since the P1 area contains one of the cell attachment sites of laminin [29, 60] and bears the growth factor-like properties of this large glycoprotein [24, 61], it is possible that the in vitro inhibition of neurite outgrowth is due to poor attachment and/or survival caused by antibodies interfering with these domains. Alternatively, the conformation of pure laminin attached to the culture substratum may be such that antibodies directed against a cell attachment region could be close enough to the neurite outgrowth domain to cause steric hindrance and therefore impair attachment and neurite outgrowth. The glial form of laminin may be deposited on the cell surface in a way that keeps antibodies from interfering with the neurite outgrowth function of laminin. Furthermore, living glial cells secrete and deposit many other growth factors [84] and cell adhesion molecules [69] that can support neuronal survival and axon growth [58, 74].

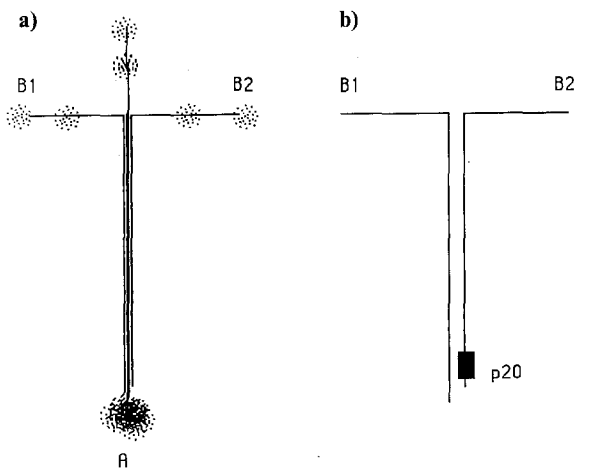

Figure 3. Schematic comparison of the basement membrane *(a)* and glial *(b)* laminins as suggested by biochemical data. In *(b)* a neurite outgrowth domain (p20;52) is shown in its putative location.

Usefulness of functional studies using antibodies to analyze the role of extracellular matrix proteins in the nervous tissue depends on the following: 1) the antigenic site that the antibody recognizes; a large protein may have many such domains; 2) the similarity between the protein used to produce antibodies and the protein present in the tissue; 3) whether the binding of the relatively large immunoglobulins and even their Fab fragments masks the actual antigenic site. Therefore, negative results with antibodies should not be considered as a final proof and positive results should be considered as suggestions. Identification of the neurite outgrowth promoting domain of laminin offers a good example: Antibodies that specifically bind to the heparin binding domain of laminin were originally used to localize a neurite outgrowth domain of laminin in the globular end of the A chain [20]. However, preabsorption of laminin-coated substrata with heparin did not interfere with binding of neurons to laminin (Liesi, unpublished data). Since biochemical data indicated that the glial laminin is a variant form with the A chain missing [50], it seemed unlikely that the heparin binding domain of the A chain could be responsible for the neurite outgrowth effect of laminin for mammalian central neurons. It has also been shown that Schwann cells produce laminin without a biochemically detectable A chain [17, 21], and it is now generally agreed that the globular end of the A chain is not the actual neurite outgrowth domain but that this domain is localized close to the neurite outgrowth promoting area. Therefore antibodies that bind to the heparin binding domain can interfere with binding of neurons to laminin.

The involvement of basement membranes in neuronal movement is at present a controversial issue. Some investigators have shown dramatic neurite outgrowth on isolated basement membranes [19], while others have concluded that basement membranes do not support neurite outgrowth [67].

In vivo, neurites or migrating neurons are not normally in touch with basement membranes. This is the case even in the optic nerve in which the neurites grow close to but never in direct contact with the basement membrane. It is possible that different basement membranes support neurite outgrowth differently. This may be suggested, because laminin(s) have during the past few years turned out to be a large family of homologous proteins [4] that have different tissue distributions and putative functions. It is therefore possible that in some basement membranes the interactions of laminin with other proteins could be such that neurite outgrowth is supported and in others such that it is inhibited. This might function in vivo to create areas that favor and others that inhibit growth, which might be of relevance in injured brain.

Fibrillar matrix of laminin does not support neurite outgrowth in vitro [16, 41, 67]. This has also been demon-

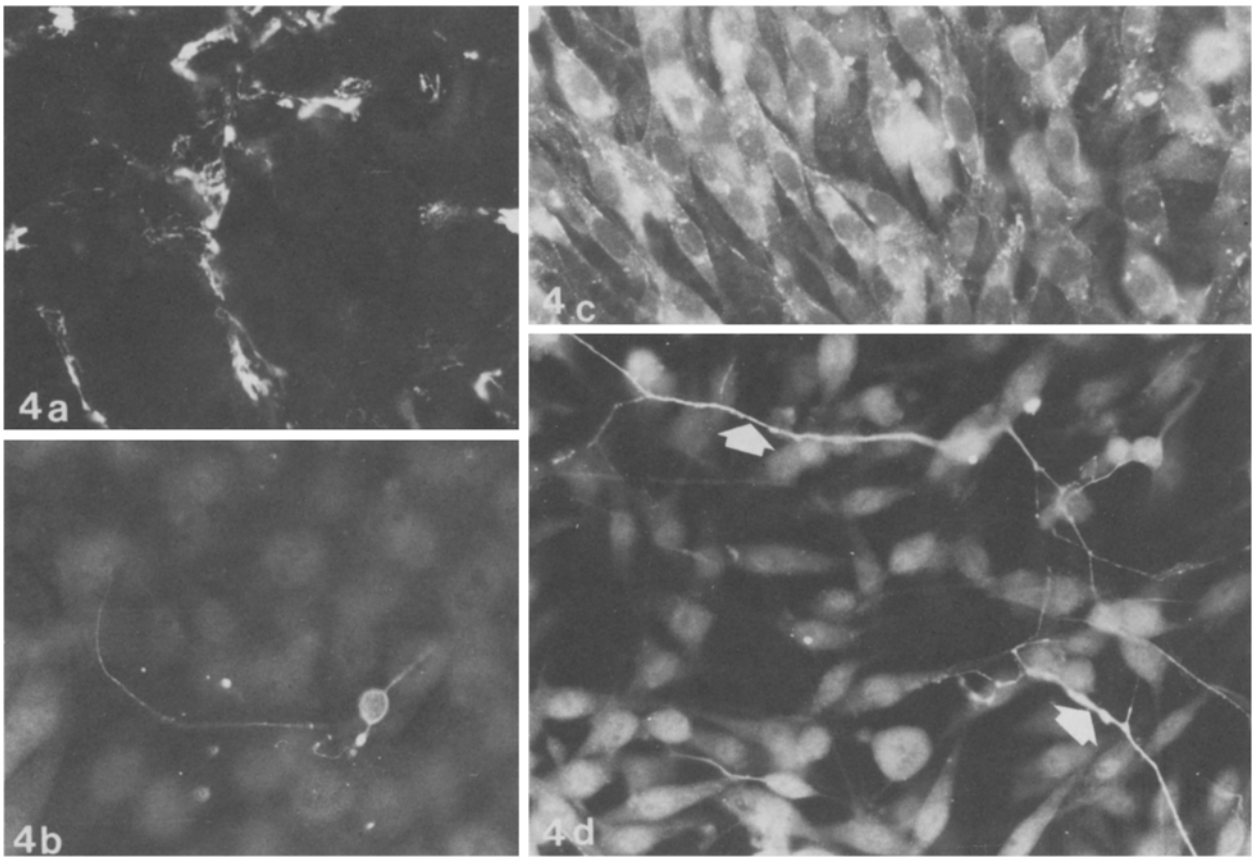

Figure 4. Immunostaining of laminin *(a, c)* and neurofilament proteins *(b, d)* in cocultures of rat central neurons and glioma cells. A glioma cell line that deposits laminin in a fibrillar matrix *(a)* provides a poor substratum for neurite outgrowth *(b)*. Another glioma cell line that assembles laminin in extracellular punctate deposits *(c)* supports neurite outgrowth (*d*; arrows).

strated in vivo in situations where scar formation inhibits neurite outgrowth in adult brain injuries[64] or in experimental situations in which pieces of nitrocellulose have been implanted in old mammalian brain tissue, which does not support neurite outgrowth[73]. Our recent studies show that cultured glioma cells that deposit laminin in the form of extracellular matrix as opposed to punctate deposits provide a poor substratum for neuronal attachment and neurite extension (fig. 4). Aged glial cells synthesize an extensive fibrillar laminin matrix in vitro[67]. Since this matrix can not be created by laminin alone[30] the fibrillar deposits may be an in vitro analogue of the basement membrane. Indeed, at electronmicroscopic level this glial scar contains basement membrane like structures[67]. Furthermore, other basement membrane components, such as fibronectin[48] and the basement membrane heparansulfate proteoglycan[2] codistribute with the fibrillar form of laminin in vitro.

The laminin gene family: the putative role of its members in neuronal migration and axon guidance

During the past few years laminins have been found to be a family of relatively homologous proteins[4]. The basement membrane prototype, originally isolated from a mouse EHS tumor[4], has new isoforms that have been re-named to support their identity or their specific functions. Common to all of the laminins is some degree of sequence homology and the presence of a defined domain structure[4, 54]. The B chains of laminin have been cloned in mouse, human, drosophilia[4], and *C. elegans* (Hedgecock, personal communication). The A chain has been cloned in mouse[4]. The cloned B chain homologues of laminin from different species show greatest sequence homology in the amino terminal parts of the proteins[4, 37]. Biochemical evidence suggests that heart laminin is different from other laminins. It has a new chain not identified in other laminins[62]. Sea urchin has a distinct laminin[55], and leech laminin has been purified and shown to promote neurite outgrowth of leech neurons[10]. In mammals, the astrocyte laminin has also been shown to be a variant form with the A chain missing[50]. Little A chain expression has been detected during early kidney development[40]. Merosin, a newly identified protein that is specifically distributed in a subset of basal laminae[43] has been shown by molecular cloning to be an A chain variant that promotes neurite outgrowth (Leivo, personal communication). S-laminin of the mammalian neuromuscular junction is the least homologous among

the presently identified mammalian sequences to the EHS tumor laminin[37]. This laminin is also present in developing brain (Sanes, personal communication), but it is not thought to support neurite outgrowth[38]. The specific functions of extracellular matrix proteins, in particular laminin(s) will be better understood once specific isoforms have been purified and antibodies and nucleotide probes generated. However, it must be realized that since glycosylation modulates binding of proteins to laminin[10] primary structural work and recombinant protein production will still have this limitation in attempts to understand protein function.

Experimental evidence for the role of laminin in neuronal migration and axon guidance

In the chicken and in mammals, only indirect evidence is presently available to support a role of laminin in neuronal migration and neurite outgrowth in vivo. The evidence is simply based on the fact that laminin is expressed in the central nervous system in temporal and spatial correlation with neurite outgrowth and axon guidance. Antibodies against the basement membrane laminin have been inactive in interfering with nervous system development whereas a monoclonal antibody that recognizes a laminin-heparan sulfate complex[12] (INO) produces defects in chicken neural tube development[7]. Very similar defects have previously been observed when embryonic chickens were injected with another antibody which recognizes the B1 subunit of the laminin/fibronectin receptor complex[6]. These results suggest that both antibodies inhibit the glial laminin function. The function of laminin(s) in mammalian brain will be directly tested when antibodies to glial laminin and its neurite outgrowth promoting domains become available.

Research on nematodes provides the first genetic evidence for laminin's involvement in neuronal migration and axon growth: it has recently been shown that in *C. elegans* nervous system development one of the essential genes for neuronal migration and nerve pathway formation (unc 6) is a homologue of the B2 chain of laminin (Hedgecock et al., Neuron *4* (1990) 61–85). Mutation of this gene perturbs neuronal migration and growth of pioneer axons in the *C. elegans* nervous system.

In vitro studies indicate that cooperation of integrin, N-cadherin, N-CAM[58] and L1[74] antibodies is needed to interfere with neuronal binding onto glial cells in vitro. This indicates the involvement of multiple adhesion mechanisms in neuronal-glial interactions. Importantly, Smith et al. have further shown that the ability of these antibodies to inhibit neuronal attachment and neurite outgrowth on glial cells is dependent on the age of the astrocytes in vitro. Only the young glial cells seem to function via these interactions whereas neurite outgrowth on older glial cells is much less influenced by these antibodies[74]. It is known that glial cells stop making laminin when they age in culture[44] and in vitro studies have implied that this is accompanied by initiation of fibronectin synthesis[48,63]. Since fibronectin promotes outgrowth of short neurites of central neurons (fig. 1), it might be responsible for the neurite outgrowth response of neurons on aged glial cells in vitro. Such neurite outgrowth would then be largely independent of N-CAM, L1, N-cadherin and the integrin B1 subunit, and might occur via neuronal receptors more specific for fibronectin.

Are the extracellular punctate deposits of laminin an artifact?

In most recent papers on neurite outgrowth in vivo[71] laminin is demonstrated as punctate deposits. Punctate deposits have been demonstrated earlier along the radial glial fibers during the migratory phase of developing neurons[46]. During kidney development similar punctate deposits have been associated with the condensation of mesenchyme[22]. It has been argued that such punctate deposits of laminin may be a staining artifact, or that they are not extracellular but part of the laminin staining of the rough endoplasmic reticulum seen in cryostat sections of the mammalian brain. However, the extracellular nature of these punctate deposits is evident in the chicken optic nerve in which laminin can be demonstrated only in this form and no immunoreactivity is seen in the cell bodies that presumable synthetize it[15]. Since antibodies used in these studies are against mouse laminin, it is possible that interspecies cross reactivities do not allow demonstration of the cellular pool of laminin in the chicken optic nerve. The other alternative is that in the chicken most of the laminin is rapidly secreted and perhaps not stored in the glial cells as is the case in the mammal. Antigenic differences may further explain the fact that no laminin is seen in punctate deposits in frog or gold fish brains but the protein appears in glial cells as homogeneous accumulations[47].

Immunoelectron microscopy has confirmed the presence of laminin as extracellular deposits on immature neuroepithelial cells in vivo[32,46]. The presence of laminin on the glial cell surfaces has also been demonstrated in vitro by immunostaining of living glial cells or cells without permeabilizing their membranes[16,49]. If the punctate deposits of laminin were a staining artifact, we might expect to see any antigen stained in a similar fashion. This is not the case; no fibronectin or type IV collagen has been demonstrated in this form[49].

Is the function of these extracellular punctate deposits of laminin to promote neurite outgrowth? This question has no definitive answer, but data presently available indicates that this could be the case: 1) Neuronal migration and axonal guidance correlate with extracellular punctate deposits of laminin. 2) Glial cells that express laminin in the punctate form support neurite outgrowth of cultured neurons far better than cells that deposit laminin in the fibrillar form[16,41,67] (fig. 4). 3) In the

optic nerve of albino rodents laminin is present in punctate deposits in the dorsal pole of the stalk area, which may be one of the reasons why axons also grow in this location (Liesi and Silver, unpublished results). 4) Antibodies raised against a neurite outgrowth domain of laminin stain the glial cell surfaces in a punctate manner (Liesi, unpublished results). It is not presently known how the punctate deposits of laminin are formed. However, since laminin interacts with several matrix proteins it is possible that either molecular interactions or self-aggregation may regulate the punctate formation[4]. It is by no means excluded that the laminin receptors on the glia could be responsible for this interaction.

The neurite outgrowth function of laminin can be mediated by short peptides

Synthetic peptides have recently been used to identify neurite outgrowth promoting domains of the basement membrane laminin molecule: Two different domains have been identified: one of them is in the alpha-helical region of the B2 chain[51] and the other one has been mapped to the carboxy terminal end of the A-chain[79]. Interestingly, the two sequences are in such close proximity, that they are likely to cooperate in basement membrane laminin in promoting neurite outgrowth. However, none of these sequences alone or in artificial combinations promote neurite outgrowth as well as laminin[51,79], and it is therefore possible that there are additional sequences in the laminin molecule that are needed to provide full support for the neurite outgrowth. Since laminin also has growth factor-like properties in the sense that it is mitogenic[61] and supports short-term survival of central and peripheral neurons[24,66], it is further possible that synthetic peptides can never fully substitute this large molecule. Lastly, it has been shown that glial laminin is a variant form[50] and it is therefore possible that the neurite outgrowth response on synthetic peptides derived from the basement membrane laminin differs from that on the glial cells because of slight but important sequence differences.

Both laminin and the neurite outgrowth domain of the carboxy terminal end of the B2 chain[51] have recently been shown to promote neurite outgrowth in soluble form[51]. This observation is in contrast to the general belief that laminin promotes neurite outgrowth when bound to the substratum. Since fragments of laminin have been detected in the cerebrospinal fluid of adult mice[65] and since small peptides of laminin are present in human sera (Liesi and Risteli, unpublished), the functions of laminin and its peptides may be more diverse than has been thought. One can speculate that the proteolytic enzymes in the developing brain may release functionally active small peptides that might influence neuronal migration or axon growth. Thus, such peptides might act as soluble guidance cues and be part of the molecules shown to control neuronal directionality[34,35,80].

The proteolytic machinery of the brain involved in degrading laminin and other matrix proteins may include plasminogen activators and some other components[57]. Laminin is known to bind to tissue type plasminogen activator (tPA)[70] and Thy-1 antigen[52]. Thy-1 also binds pro tPA with high affinity, but shows no binding to urokinase. These interactions occur without interfering with the active site of tPA which is therefore left free to interact with other proteins[52]. Since both Thy-1[52] and tPA[82] are associated with cell membranes and known to be expressed during brain development these interactions may function in binding and/or detaching laminin from the cells to allow cell movement necessary for neuronal migration and axon guidance.

Conclusion

Glial cells in the embryonic brain produce laminin in spatial and temporal correlation with neuronal migration and axonal guidance. Punctate deposits of laminin rather than its fibrillar matrix form may be growth stimulatory. Neurite outgrowth promoting domains of the basement membrane laminin have been identified using synthetic peptides. These studies imply that laminin promotes neurite outgrowth in both soluble and substrate bound forms. Glial cells of mammalian brain synthetize a variant form of laminin. Molecular interactions of the glial laminin with glycosaminoglycans and remaining matrix proteins may regulate the earliest phases of neuronal movement.

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Mechanisms of glial-guided neuronal migration in vitro and in vivo

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Summary. Our laboratory has developed an in vitro model system in which glial-guided neuronal migration can be observed in real time. Cerebellar granule neurons migrate on astroglial fibers by apposing their cell soma against the glial arm, forming a specialized migration junction, and extending a motile leading process in the direction of migration. In vitro assays indicate that the neuronal antigen astrotactin functions as a neuron-glia ligand, and is likely to play a role in the movement of neurons along glial fibers. In heterotypic recombinations of neurons and glia from mouse cerebellum and rat hippocampus, neurons migrate on heterotypic glial processes with a cytology, speed and mode of movement identical to that of neuronal migration on homotypic glial fibers, suggesting that glial fibers provide a permissive pathway for neuronal migration in developing brain. In vivo analyses of developing cerebellum demonstrate a close coordination of afferent axon ingrowth relative to target cell migration. These studies indicate that climbing fibers contact immature Purkinje neurons during the migration and settling of Purkinje cells, implicating a role for afferents in the termination of migration.
Key words. Neuronal migration; astrotactin; neuronal antigen; heterotypic recombination; glial-guided migration.

Introduction

Neuronal migration has traditionally been viewed as a discrete step in CNS development, separated in time from preceding periods of neuronal proliferation in ventricular zones and from subsequent periods of axon ingrowth into neuronal layers of the cortex [38, 55]. Although the role of astroglia in providing a cellular substrate for migration is well established [8, 10, 12, 19, 45–52, 55], the hierarchical roles of neuron-neuron interactions in terminating glial-guided migration have not been addressed. To examine the mechanism of glial-guided neuronal migration, we have developed an in vitro model system [19]. Initially we focused on the migration of the granule neuron in the developing mouse cerebellum, using in vitro systems to define the mode of neuronal movement along glial fibers [8, 12, 20], the regional specificity of migration [11] and the molecular mechanism of migration [9]. To examine