Developmental loss of functional laminin receptors on retinal ganglion cells is regulated by their target tissue, the optic tectum

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Summary

The ability of chick retinal ganglion cells (RGCs) to extend neurites on tissue culture substrata of the extracellular matrix protein laminin is lost during embryonic development. In order to establish the mechanism responsible for the loss of response, the number of high affinity ($K_d \sim 10^{-9}$ M) laminin receptors on both the cell bodies and neurites of RGCs were determined throughout this period by a ligand binding assay using radio-labelled laminin. It was found that the loss of response paralleled a decrease in receptor numbers on both the cell bodies and the neurites of the RGCs. Bilateral tectal ablation at embryonic day 6 resulted in the subsequent maintenance of laminin-stimulated neurite outgrowth, together with a partial inhibition of the loss of laminin receptors. Thus, the loss of response of the RGCs to laminin reflects a decrease in the numbers of laminin receptors on these neurons, and furthermore, this down-regulation is in turn dependent on innervation of the target tissue.

Key words: laminin, laminin receptors, neuron, retinal ganglion cell, optic tectum, neurite, chick embryo.

Introduction

Laminin is a large noncollagenous glycoprotein of the extracellular matrix (Timpl et al. 1979). In addition to acting as a good tissue culture substrate for the attachment and spreading of many cell types (Martin and Timpl, 1987), one of the most potent effects of laminin is to stimulate the growth or regeneration of neurites from a variety of neuronal cells in vitro (Baron-Van Evercooren et al. 1982; Martin and Timpl, 1987). Although generally confined to the basement membranes of adult tissues, laminin immunoreactivity is also seen transiently on the membranes of neuroepithelial cells in the optic nerve (Cohen et al. 1987) and other parts of the developing central nervous system (for examples see McLoon et al. 1988; Liesi and Silver, 1988; Letourneau et al. 1988), in a pattern that prefigures the route taken by growing axons. It is therefore possible that laminin promotes the growth of axons during development in vivo.

It has recently become apparent that the neuronal response to laminin is specifically regulated during development, the ability of laminin to stimulate neurite outgrowth from chick retinal ganglion cells (RGCs) being lost as they mature between E6 and E11 (Cohen et al. 1986). In contrast, the cell adhesion molecules on the membranes of astrocytes are able to stimulate neurite outgrowth throughout this period (Cohen et al. 1986; Tomaselli et al. 1986; Neugebauer et al. 1988). While coinciding broadly with the time during which the RGCs innervate their target tissue, the optic tectum (Rager and Rager, 1978), it is not clear if the loss of response to laminin is evoked epigenetically by the tectum, or, alternatively, if the timing of the loss is cell autonomous.

In order to elucidate how the response of RGCs to laminin is regulated during development, it is necessary to establish the molecular mechanisms by which laminin interacts with these neurons. Initial attempts to identify a molecule that functions as a neuronal laminin receptor showed that laminin-induced neurite outgrowth from RGCs (Cohen et al. 1987) and other neurons (Bozyczko and Horwitz, 1986) can be blocked by antibodies directed against the integrins, a family of integral membrane protein dimers that are involved in a variety of cell–cell and cell–matrix interactions (Hynes, 1987). However, the unavailability of antibodies specific for a laminin-binding integrin has meant that it has not been established if the developmental regulation of the RGCs' response to laminin occurs at the level of the
receptor molecule (e.g. by a down-regulation of receptors). Recently, however, it has proved possible to analyse the interaction of laminin with its cell receptors directly, using a ligand-binding assay. Such assays obviate the need to have identified a specific laminin receptor molecule, and so could be used to demonstrate that the response of a variety of cell types to laminin reflects the presence of specific high-affinity ($K_D$ $10^{-9}$ M) receptors on the cell membranes (Aumailley et al. 1987; Nurcombe et al. 1989; Goodman et al. 1989).

Here we show that the developmental decline in the neurite outgrowth response of chick RGCs to laminin corresponds to a loss of high-affinity laminin receptors from their cell bodies and neurites. Furthermore, this down-regulation of receptors is shown to be an epigenetic phenomenon, the effect of tectal ablation prior to target encounter by the RGC axons being both to partially inhibit the loss of receptors, and to maintain the neurite outgrowth response of the RGCs to laminin.

Parts of this work have already appeared in abstract (Cohen, 1987; Edgar and Nurcombe, 1988).

**Materials and methods**

**Isolation of ganglion cells from embryonic chick retina**

Retinal cells from embryos of ages given were dissected free from pigmented epithelium, choroidal and scleral tissue, and incubated at 37°C for 25 min in calcium- and magnesium-free phosphate-buffered saline (PBS) containing 1 mg ml$^{-1}$ trypsin (Worthington) and 3 mg ml$^{-1}$ bovine serum albumin (BSA). The tissue was then washed in F14 culture medium (Gibco) containing 10% heat-inactivated foetal calf serum and 2% chick embryo extract, together with 100 µg ml$^{-1}$ streptomycin sulphate and 100 IU ml$^{-1}$ penicillin. In order to stop the proliferation of non-neuronal cells, the medium was supplemented with 10 µM fluorodeoxyuridine and 10 µM-uridine for the first day of culture, and thereafter with 10 µM-cytosine arabinoside. This treatment with cyto-statics prevented the outgrowth of non-neuronal cells from the explants, which were monitored by phase-contrast microscopy. Under these conditions it has been shown that the neurites growing out of the explants are derived from the RGCs (Halfter et al. 1981). Retinal explants were excised using a scalpel blade after 2 days culture for E5 and E7 embryos, and after 3 days for those from E9 embryos. The medium was then exchanged with Ca$^{2+}$- and Mg$^{2+}$-free Krebs–Ringer–Henselheit (KRH) buffer containing 1 mM EDTA at 4°C, and the RGC neurites removed from the substrate by gentle trituration. Aliquots of the neurite suspension were taken for protein determination (Lowry et al. 1951), the rest being harvested by centrifugation (10 min at 600 g) for the laminin-binding assay.

**Tectal ablation**

Bilateral tectal ablation of chick embryos at E6 was performed by dissecting out the entire midbrain tectum. An opening was made with forceps in the broad end of the egg above the air space, and the underlying shell membrane wetted with saline and then carefully removed. The embryo was manoeuvred by means of the amniotic membranes so that the tectum was accessible and could be removed with forceps without major haemorrhaging of the brain. The opening in the shell was resealed with tape and the egg returned to the incubator. Surviving embryos (>60%) were removed from the egg at E9–11 and the retinae dissected for dissociation and culture of the cells (Cohen et al. 1986), or for isolation of RGCs for laminin-binding assay as described below.

**Laminin-binding assay**

The principle of the ligand-binding assay is to separate bound from free laminin after equilibrium has been attained, by the centrifugation of the cells or neurites through a sucrose density gradient (Aumailley et al. 1987). The methods for iodination of laminin with lactoperoxidase and for the ligand-binding assay for laminin receptors on perikarya have been described in detail previously (Aumailley et al. 1987; Nurcombe et al. 1989). In addition, preliminary experiments were carried out to show that the neurites collected from retinal
Explant cultures could be resuspended in Krebs-Ringer-Henselheit buffer containing 0.1% bovine serum albumin (KRH-BSA) by gentle trituration, and then used to determine specific laminin binding in the same assay system. Purified RGCs from the upper metrizamide interface and other retinal cells from the lower interface were pelleted by gentle centrifugation (10 min, 100g) and resuspended in KRH-BSA. The 125I-labelled laminin equilibrium binding studies employed aliquots of 10^5 RGCs. Unspecific binding was determined in all cases by the addition of 100-fold molar excess of unlabelled laminin at the beginning of the incubation. Maximal specific binding of laminin to both the cell bodies and neurites occurred after 90 min at 0°C (data not shown), and this time point was used to assess equilibrium binding. While receptor quantification on the perikarya is shown), and this time point was used to assess equilibrium binding.

**Immunohistochemistry**

RGCs were identified in mixed retinal cultures using a monoclonal antibody to chick Thy-1 glycoprotein, these being the only neurons that display surface Thy-1 in the developing chick retina (Sinclair et al. 1986; Sheppard et al. 1988). Retinal cultures were prepared on cover slips as previously described (Cohen et al. 1986) and incubated for 30 min with the Thy-1 antibodies (ascites fluid diluted 1:100 in DMEM containing 15mM-Hepes buffer and 10% FCS), then with biotinylated anti-mouse antibodies (Amersham) diluted 1:100 in the same medium for 30 min, and finally with fluorescein-conjugated streptavidin (Amersham) diluted 1:100 in the same medium for 30 min. The cultures were washed 3 times in medium between each incubation, and finally fixed in 95% etha-

**Results**

Loss of laminin-induced neurite outgrowth from RGCs reflects the number of high affinity laminin receptors

The isotherms of specific binding of radiolabelled laminin to suspensions of purified RGCs were used to determine the number of high affinity cellular receptors for laminin and their binding affinity. Fig. 1 shows that while the maximal amounts of specific laminin binding varied markedly for RGCs obtained from chicks of different embryonic ages, the affinity of binding remained constant (K_D 1—2×10^-7 M), as shown by the parallel Scatchard plots generated from the binding data (Fig. 1, insert). The changes seen are therefore most likely to be due to simple regulation of the numbers of functional receptors. Thus, quantification of the maximal specific laminin binding at different embryonic stages revealed that over 85% of the high affinity laminin receptors were lost from the ganglion cells, a maximum of 74000 receptors per RGC at E6 being reduced to 8000 receptors per RGC at E12, most of this decrease occurring before E8 (Fig. 2A).

In order to establish to what extent the loss of laminin-induced neurite outgrowth from the RGCs paralleled the decrease in receptor number, neurite outgrowth from RGCs of intermediate stages between E5 and E11 was examined. The results show that the previously reported loss of response to laminin (Cohen et al. 1986) also had occurred before E8 (Fig. 2B). The use of the short 10h culture period for the neurite outgrowth assay indicates that the loss of response to laminin noted previously (Cohen et al. 1986) is unlikely to have been caused by a general inability of the older RGCs to survive in culture without adequate trophic support: the viability of cells from all the ages remained high up to the end of the shorter 10h assay used here (see Materials and methods).

A much smaller decrease in receptor numbers (about 50%) was seen for retinal cells in the fraction from which most of the RGCs had been eliminated on centrifugation (Fig. 1A). Indeed, the fact that this fraction was still contaminated with RGCs (some 8% of these cells were Thy-1 +) indicates that the RGCs will make a considerable contribution to the low average numbers of receptors per cell determined in that fraction.

In contrast to the simple decrease in receptor numbers seen on the cell bodies of the RGCs, determination of the receptor content of the neurites revealed that in addition to the developmental loss of laminin-binding sites, more strikingly the apparent affinity of these sites for laminin decreased approximately 50-fold between E5 and E9, with the major decrease again occurring during the first half of this period (Table 1). Thus there seems to be an additional regulatory mechanism operating to attenuate the laminin receptors on the neurites and growth cones of the RGCs.
Table 1. Developmental decrease in the amount and affinity of specific laminin binding to RGC neurites

<table>
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<tr>
<th>Embryonic age</th>
<th>Maximum specific binding</th>
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<tr>
<td></td>
<td>(fmol laminin mg⁻¹ neurite protein)</td>
</tr>
<tr>
<td>E5</td>
<td>2.12±0.08</td>
</tr>
<tr>
<td>E7</td>
<td>0.62±0.06</td>
</tr>
<tr>
<td>E9</td>
<td>0.42±0.02</td>
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Means±s.e.m. are shown derived from three independent experiments, the neurites being collected from cultured explants of retinae taken from chick embryos of the ages shown.

Table 2. Effect of tectal ablation on the developmental decrease of RGC laminin receptor numbers

<table>
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<tr>
<th>Embryonic age</th>
<th>Receptors per cell</th>
<th>$K_D$ (nm)</th>
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</thead>
<tbody>
<tr>
<td>E6</td>
<td>74 700±2300</td>
<td>1.86</td>
</tr>
<tr>
<td>E9 (control)</td>
<td>19 800±590</td>
<td>1.98</td>
</tr>
<tr>
<td>E9 (ablated)</td>
<td>37 300±6600</td>
<td>1.68</td>
</tr>
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RGCs were prepared from E9 embryos that had undergone bilateral tectal ablation at E6, and from sham-operated controls. Ablation did not affect the yield of RGCs obtained after the purification procedure. The results shown are the means±s.e.m. of 4 control embryos and 7 experimental embryos, the data for the E6 embryos being taken from Fig. 2 for purposes of comparison. The number of receptors determined for the experimental animals differs at the $P<0.001$ level (Student $t$-test).

Discussion

Taken together, the results presented here illustrate a striking parallel between the tectum-dependent loss of laminin-induced neurite outgrowth response of RGCs and their laminin receptor numbers.

Tectal ablation maintains both the laminin-induced neurite outgrowth response of RGCs and their laminin receptor numbers

In order to determine if the optic tectum was instrumental in regulating the loss of response of the RGCs to laminin, bilateral ablation of the midbrain at E6 was performed. No obvious changes in the retina were apparent up to E11 in the tectotomised chicks, in agreement with previous reports (Hughes and La Velle, 1975), and no differences between control and operated embryos were found in the numbers of Thy-1⁺ RGCs per dissociated retina (data not shown). However, when retinal cells from E11 control and ablated embryos were cultured on laminin, there was an obvious increase in the numbers of RGCs from the ablated animals able to extend neurites (Fig. 3). Quantification of the results of 5 such experiments revealed that there was an almost 3-fold increase in the proportion of RGCs with neurites (Fig. 4).

Laminin receptor numbers on the RGC cell bodies were then determined after the maximal decrease in both response and receptor numbers had already taken place by E9 (see Fig. 1), and several days before any changes in RGCs due to tectotomy become apparent (Hughes and La Velle, 1975). The results show that the tectal ablation partially inhibited the loss of laminin receptors from the RGCs, so that approximately double the number of receptors was found on the RGCs of tectotomised chicks, relative to controls (Table 2).
Regulation of laminin receptors

Fig. 3. Neurite outgrowth on laminin substrata by RGCs taken from control (A) and tectal-ablated (B, C) embryos. The immunofluorescent staining of Thy-1 positive RGCs was photographed 24 h after the start of culture of cells dissociated from the retinas of E11 chick embryos. Bar represents 20 \( \mu \)m.

Fig. 4. Quantification of neurite outgrowth from Thy-1 positive RGCs from E11 tectal-ablated (TA) and control (C) embryos. Means±standard deviations are shown for the results from 5 embryos. The values from the tectal-ablated embryos are significantly greater than those from the controls (\( P<0.01 \)).

laminin receptors on chick RGCs and the developmental decrease in laminin-induced neurite outgrowth from these neurons in culture.

Clearly the correlation between high affinity receptor numbers and the response of the RGCs to laminin substrates does not prove a causative link. Nevertheless, we think that the receptors demonstrated by ligand-binding assay are likely to be responsible for neurite outgrowth for a number of reasons: not only does the normal developmental loss of response to laminin reflect the normal decrease in receptor numbers, but this correlation also applies to the experimental situation, prior tectal ablation helping to maintain both receptors and response. Furthermore, previous observations with a variety of non-neuronal cells have demonstrated a good correlation between presence of high affinity receptors, and the ability of the cells to attach or spread on laminin tissue culture substrates (Aumailley et al. 1987). Significantly, it was shown recently that receptor binding is via the same domains of the laminin molecule that evoke cellular responses, increasing the likelihood that the high affinity binding of laminin to cells is via the physiologically relevant laminin receptors (Nurcombe et al. 1989; Goodman et al. 1989).

One advantage inherent in the use of neurons to investigate laminin receptors is that it is possible to examine receptors from different cellular locations i.e. receptors either on the perikarya, or those located towards the extremities of the neurites that are directly involved in the laminin binding which results in the stimulation of growth cone migration. As expected, the numbers of laminin receptors determined on the neurites was also lower for older RGCs. Surprisingly,
however, there was also a marked decrease in the affinity of the neurite receptors for laminin, indicating that a hitherto uncharacterised differential regulation or modification of laminin receptors occurs between the perikarya and the neurites.

The molecular identity of the high-affinity laminin receptor remains to be established. While several laminin binding proteins have been identified in neural tissue, it has not been demonstrated that any of these function as the laminin receptor present on RGCs or other neurons (Smallheiser and Schwarz, 1987; Kleinman et al. 1988; Douville et al. 1988). Recently, however, it has been shown that integrin molecules can be extracted from the membranes of a number of cell lines (including glioblastoma, neuroblastoma and phaeochromocytoma) and will bind to laminin coupled to a solid support for affinity chromatography. (Gehlsen et al. 1988; Ignatius and Reichardt, 1988). Furthermore, monoclonal antibodies directed against a common Integrin β-subunit not only block neurite outgrowth from RGCs on laminin substrates (Cohen et al. 1987), but can also inhibit the high affinity binding of laminin to RGCs (own unpublished observations). An earlier direct attempt to detect an integrin or associated protein that might be responsible for developmentally regulated laminin–cell interactions was unsuccessful (Hall et al. 1987), possibly because total populations of retinal cells were investigated, where any decrease in a laminin-specific component would be expected to be smaller than that of the RGCs: a much smaller decrease in receptor numbers (about 50%) was seen for retinal cells in the fraction from which most of the RGCs had been eliminated on centrifugation. If the neuronal laminin receptor should, however, turn out to be an integrin complex, then one consequence of development would be the independent regulation of expression of individual integrin subunits: the amount of a common integrin β-subunit detectable on the surface of RGCs apparently increases during development (Cohen et al. 1987), although a decrease in laminin receptors and response occurs, presumably via the regulation of a laminin-specific integrin α-subunit. It remains to be determined if either of the two presently described integrin complexes with distinct α-subunits reported to interact with laminin also mediate the neuronal response to laminin (Sonnenberg et al. 1988; Takada et al. 1988).

The timing of the developmental loss of both receptors and neurite outgrowth response of RGCs to laminin shown here coincides with their innervation of the optic tectum (Rager and Rager, 1978), and it has been noted previously that the ability of other types of neurons to extend neurites on extracellular matrix molecules is lost during development, also coinciding with target innervation (for example, see Collins and Lee, 1982). It is unlikely that any damage to RGC axons occurring as a side effect of the tectal ablation is responsible for the maintenance of receptors and response shown here: the number of RGC axons immediately distal to the orbit at E6 represents only some 3% of those present between E9 and E11 (Rager and Rager, 1978), whereas tectotomy at E6 resulted in maintenance of the neurite outgrowth response of some 40% of the RGCs at E11. Thus we conclude that receptor loss during development may be regulated epigenetically, in this case being dependent on the presence of the neuronal target tissue. It remains to be seen if the regulation of laminin receptors on other cell types (Goodman et al. 1989) is epigenetic or cell-autonomous.

The mechanism by which a target tissue regulates laminin receptor expression by its innervating neurons remains unknown. Such tissues play a profound role in the development of the nervous system, target-derived neurotrophic factors being necessary for neuronal survival (see Davies et al. 1987), and other molecules found in the targets (possibly including laminin) affecting the differentiation of neural cells (Baron-Van Evercooren et al. 1982; Acheson et al. 1986; Dohrmann et al. 1986). Somewhat paradoxically, it has recently been shown that the survival and regeneration of cultured adult rat RGCs is stimulated by the brain-derived neurotrophic factor, BDNF (Thanos et al. 1989). If such molecules should also be involved in the developmental loss of laminin receptors, then clearly the responses they elicit differ markedly between development and regeneration.

We conclude that the response of chick RGCs to laminin is determined by the expression of high-affinity laminin receptors on these cells, the down-regulation of the receptors during development being in turn determined epigenetically by innervation of the target organ of the RGCs, the optic tectum. It remains to be seen whether the effect of the tectum on the expression of laminin receptors by RGCs is mediated by direct contact of the RGC axon terminals with their target, or, alternatively, if a neurotrophic molecule is involved.

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