Spatial and temporal variation in the structure of the basal lamina in embryonic grasshopper limbs during pioneer neurone outgrowth

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Summary
The pioneer neurones of the embryonic grasshopper limb use the basal lamina underlying the limb ectoderm as a substratum over which to grow from the periphery to the CNS (Anderson & Tucker, 1988). In this paper we use transmission electron microscopy to describe the structure of this substratum before, during, and after the time of axon navigation.

The organization of the basal lamina varies considerably in different regions and at different times of development of the embryonic limbs, and is unlike that of the fully developed limb at the time of hatching. We suggest that this spatial and temporal variation could play a role in regulating the direction of outgrowth of pioneer neurones.

Key words: basal lamina, gradients, axon guidance, pioneer neurones, neurone substratum, grasshopper embryo.

Introduction
The embryonic grasshopper limb provides a relatively simple preparation for studying the complex problem of how neurones interact with surrounding tissues to navigate with precision to their targets. At 30% of embryonic development a single pair of neurones, the Tibia 1 (T11) neurones, begins to grow in a characteristic trajectory through the limb which at this stage is only a tube of ectoderm containing scattered mesodermal cells. The T11 neurones are commonly referred to as pioneer neurones since they are the first neurones to appear within the limb and form the first axon pathway between the limb and the CNS (Bate, 1976). Transmission electron microscopy of pioneer neurones injected with horseradish peroxidase at different stages of outgrowth has shown that their growth cones and associated filopodia are frequently in contact with extracellular matrix material in the form of a basal lamina underlying the ectoderm (Anderson & Tucker, 1988).

In this study we use transmission electron microscopy to examine basal lamina structure in the limbs before, during, and after the time of pioneer axon outgrowth. We show that the basal lamina is unlike that of the fully developed limb at the time of hatching. Its structure also varies considerably in different regions and at different times of development of the embryonic limb. We suggest that this spatial and temporal variation could play a role in regulating the direction of outgrowth of pioneer neurones.

Materials and methods

Embryos
Schistocerca americana eggs were collected from a colony maintained at Davis under standard conditions. Eggs were immersed in saline (Shankland et al. 1982) and the embryos dissected free from the egg case, yolk, and surrounding membranes, using fine forceps and scissors. The embryos were staged according to Bentley et al. (1979).

Electron microscopy
(i) Fixation, staining, and embedding
Embryos at 30%, 35%, and 40% of development and at the time of hatching were fixed using methods modified from Bastiani et al. (1984). The embryos were fixed for 1 h on ice in 2% glutaraldehyde, 2% paraformaldehyde, 0.1% tannic acid (Mallinckrodt) and 0.25% dimethyl sulphoxide in 0.1M-sodium cacodylate buffer, pH7.4. Specimens were then rinsed twice in buffer, twice in distilled water, stained en bloc for 1 h in 2% uranyl acetate (aqueous), rinsed in distilled water, and finally stained in 1% tannic acid (aqueous) for 1h at room temperature. Embryos were then rinsed in distilled water, dehydrated in a graded ethanol series, cleared for 2 min each in 2 changes of 1:1 ethanol: propylene oxide, followed by 2 min each in 2 changes of pure propylene oxide, and embedded in Araldite.
Thick and thin sectioning
Specimens were oriented to yield either cross sections or longitudinal sections through the limb buds, and sectioned at 8 \( \mu \text{m} \) on a Sorvall MT-2 microtome. Thick sections were placed individually onto drops of distilled water on siliconized slides, dried, and then observed using darkfield optics on a Zeiss standard microscope. Appropriate sections were photographed and reembedded by inverting a BEEM capsule filled with Araldite onto the section. After the Araldite was cured, the reembedded section was removed from the slide, trimmed, and thin-sectioned with a DuPont diamond knife on a Reichert OmU3 ultramicrotome. Thin sections were stained in 2% uranyl acetate and Venable's lead citrate (Venable & Coggeshall, 1965), and observed at 60 kV with a Philips 410 transmission electron microscope. The precise positions of electron micrographs were recorded on enlargements of the photographs of thick sections.

Limb tip amputations
Differences in the morphology of the basal lamina at different proximal–distal levels of the embryonic appendages could be due to incomplete penetration of fixatives and stains into the lumen of the appendage from the body proper (which is open to the surrounding medium on the dorsal surface). To test this possibility, two sets of experiments were performed to permit better access of solutions to different regions of the limb: immediately prior to fixation, the tips of 35% thoracic limb buds were either ablated near the tibia/tarsus boundary with tungsten needles and the exposed stumps were processed, or the tips were removed near the femur/tibia boundary and the exposed tips were processed in parallel with limbs which were left intact and attached to the body. Processing for electron microscopy was as above.

Scanning electron microscopy
Some embryos were prepared for scanning electron microscopy. Specimens were fixed in 2% glutaraldehyde in 0.1 M-sodium cacodylate buffer for 1 h at room temperature, rinsed in distilled water, dehydrated in ethanol, and critical-point dried with CO\(_2\) (Samdri-780A, Tousimis). Embryos were then mounted, sputter-coated (Denton) with gold, and observed at 20 kV in a Philips 501 scanning electron microscope.

Results
The general arrangement of the fully segmented limbs of a 40% grasshopper embryo is shown in Fig. 1A. The nomenclature used for the limb axes, and for the segments of the limb is shown in Fig. 1B. The segments from tip to base are the tarsus, tibia, femur, trochanter and coxa. The pathway taken by the pioneer neurones within the limb is also shown in Fig. 1B. The pioneer cell bodies lie in the tibia. Their axons pass proximally, along the dorsal side of the limb, through the femur to

Fig. 1. The arrangement of limbs and their pioneer neurone pathways in the 40% grasshopper embryo viewed from the ventral side. (A) SEM of a 40% embryo. pr, prothoracic limb; ms, mesothoracic limb; mt, metathoracic limb. (B) Diagram of the pioneer neurone pathway in the metathoracic limb, based on a camera lucida drawing. d, dorsal; v, ventral; a, anterior; p, posterior; e, ectoderm; Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus; T11, the pair of pioneer neurones and their pathway. Bar, 0.15 mm (A), 0.2 mm (B).
the trochanter. There they turn and pass circumferentially under the anterior side of the limb to the opposite, ventral, side of the limb. They then cross the segment border into the coxa and pass proximally near the ventral edge of the coxa to the CNS.

Pioneer growth cones and associated filopodia are frequently in contact with basal lamina (Anderson & Tucker, 1988). To investigate whether the basal lamina in the region of the pioneer pathway differed from basal lamina elsewhere in the limb, we examined the structure of basal lamina in different limb regions before, during, and after pioneer neurone outgrowth. The following results are based on observation of sections through the limbs of 11 embryos and one newly-hatched larva. A total of 35 thick sections from at least 3 embryos each at stages 30%, 35%, and 40% of development were reembedded, thin-sectioned, and observed in the transmission electron microscope. For each timepoint, representative electron micrographs are presented, and their region of origin indicated on a scanning electron micrograph of the limbs. In all electron micrographs, the ectoderm is to the left and the lumen of the limb to the right. The basal lamina is indicated by small arrows.

The term basal lamina is generally used to refer to a single mature structure composed of a lamina densa and a lamina lucida, together about 150 nm thick. The lamina densa is electron dense and is composed of fibrillar and granular material. The lamina lucida lies between the lamina densa and the plasma membrane and is mostly electron transparent but may contain fine strands of material that extend from the plasma membrane through the lamina lucida to the lamina densa (Reale, 1984). The basal lamina of the developing grasshopper limb differs considerably from this standard. Measurements of basal lamina thickness in this study therefore refer to the thickness of the electron-dense material only. The basal lamina is indicated by small arrows on all of the electron micrographs presented here.

30% embryos

At 30% of development, the pair of pioneer neurones has been formed by the division of an ectodermal cell at the tip of each limb. At this stage the metathoracic limb is approximately 250 μm long, 150 μm in diameter, and has a slight ventral and anterior bend (Fig. 2A). The prothoracic and mesothoracic limbs are slightly smaller (about 175 μm long and 100 μm in diameter). The limbs show no visible sign of segmentation (Fig. 2A).

At the base of the mesothoracic limb, in the region of the future coxa, there is a basal lamina tightly apposed to the ectoderm (Fig. 2B). The basal lamina is a single continuous sheet, is relatively thick (100–150 nm) and is electron dense (Fig. 2B). It is festooned with electron-dense granules 30–60 nm in diameter (Fig. 2C). The morphology of the basal lamina at the base of the metathoracic limb is similar.

More distally, approximately one-third the length of the limb from the base, in a region that will later develop into the trochanter or proximal femur, the ectoderm is underlain by a basal lamina that is also continuous but less thick (50–120 nm), and has fewer granules (Fig. 2D,E) than basal lamina at the base of the limb (Fig. 2B,C). In the dorsal portion of the limb, the basal lamina is multilayered (Fig. 2D), whereas in ventral regions the basal lamina is usually a single sheet that is closely apposed to the ectoderm (Fig. 2E).

Approximately two-thirds the distance from the base to the tip, in a region that corresponds to the future femur of the metathoracic limb, the general morphology of the basal lamina is similar to that in more proximal regions, with a typical thickness of 50–100 nm (Fig. 2F). The basal lamina along the dorsal part of the limb is multilayered (Fig. 2F), although elsewhere around the circumference at this level it is usually a single sheet.

At the tip of the metathoracic limb, the basal lamina is discontinuous, and is very thin (30–40 nm), and often it is not in contact with the overlying ectoderm (Fig. 2G,H). It contains scattered granules and frequently bears fibrillar branching structures (Fig. 2H).

35% embryos

By 35% of development, the limbs are clearly bent towards the midline and the limb segments are externally visible (Fig. 3A). The metathoracic limb has grown to approximately 350 μm in length (Fig. 3A) and the cell bodies of the pioneer neurones are no longer at the tip. The pioneers have formed axons which have grown as far as the central nervous system.

The basal lamina around the entire base of the limb is electron dense and multilayered (Fig. 3B,C). It varies in thickness from 70 to 100 nm (Fig. 3B,C). It has a thin fibrillar core coated with many electron-dense granules approximately 30–40 nm in diameter.

More distally, in the region of the future trochanter and proximal femur, the basal lamina exhibits considerable regional variation around the circumference of the limb. Dorsally, the basal lamina is multilayered, electron-dense (Fig. 3D) and thick, ranging in thickness from 200 nm to 300 nm. In places, the basal lamina is not in contact with the overlying ectoderm. Along the posterior surface, the basal lamina is a single sheet and is considerably less electron dense than in dorsal regions (Fig. 3E). This basal lamina is closely apposed to the ectoderm, and is uniformly 70 nm thick. As one moves ventrally, the basal lamina becomes more electron dense and considerably thicker (up to 150 nm; Fig. 3F). In places, this basal lamina appears to split into two sheets each approximately 70 nm thick. The basal lamina on the anterior surface of the limb becomes progressively more multilayered as one moves dorsally. Up to six layers are found on the dorsal surface.

Near the tip of the limb, in the developing tibia, the basal lamina is usually a single sheet, but dorsally, multilayered structures are seen (Fig. 3G).

At the tip of the limb, in the developing tarsus, the basal lamina is thin and patchy (Fig. 3H).
Spatial and temporal variation in basal lamina structure

40 % embryos
At 40 % of development, the metathoracic limb is approximately 450 μm in length (Fig. 4A). The limbs have bent medially so that their distal tips nearly meet at the ventral midline. Segment boundaries are more pronounced than at earlier stages.

The basal lamina in the proximal segments (coxa and trochanter) is patchy and occasionally absent altogether. This is especially apparent in the dorsal part of the limb, where mesoderm from the base of the limb bud appears to be advancing distally between the ectoderm and the basal lamina, displacing the basal lamina from the ectoderm (Fig. 4B).

The basal lamina at the trochanter/femur border remains intact, is multilayered, and is usually closely associated with the ectoderm (Fig. 4C). The basal lamina in the femur is thick, and also usually multilayered (Fig. 4D), but in at least one specimen a single, thick (125 nm) basal lamina was observed in the mid-dorsal region of the femur.

In the tibia, which is nearly as long as the femur at this stage, the basal lamina is also usually multilayered, with 2 to 8 sheets of matrix material seen (Fig. 4E,F). Each sheet tends to be thin (30–40 nm thick), unlike the sheets in the femur (Fig. 4C,D) which frequently appear to be coated with granular material.

In the tarsus, at the tip of the limb bud, the basal lamina is thin (10–30 nm) and discontinuous (Fig. 4G).

Limb-tip amputations
We considered it possible that the differences in the morphology of the basal lamina in different regions of the limb result from uneven penetration of fixatives and stains from the base of the limb. Two sets of experiments were performed to test this possibility: the tarsi of some embryos were ablated just prior to fixation, so that fixatives and stains could also enter the limbs from the tip; in others limb tips were removed near the femur/tibia boundary and processed in parallel with limbs remaining attached to the body.

The basal lamina adjacent to the ablated region was very electron dense (Fig. 5A). This basal lamina was approximately 60 nm thick. Just 15 μm proximally (about 3 cell diameters) the basal lamina was considerably less electron dense, but remained approximately 60 nm thick (Fig. 5B). 100 μm from the wound, in the region of the femur, the basal lamina was considerably thicker (100–130 nm; Fig. 5C), though less electron dense than the more distal basal lamina. The thickness and appearance in all respects other than electron density were the same as in the intact limb.

The basal lamina in the most distal region of the tips which had been removed and exposed to fixatives and staining solutions near the femur/tibia boundary, was indistinguishable from the basal lamina in the same region of limbs that had remained attached to the body during processing – the basal lamina was thin and discontinuous (Fig. 5D,E).

Thus regional variation in the electron density of basal lamina could result from incomplete penetration of stains from the base of the limb in intact embryos, but variation in the thickness or structure of the basal lamina within the limb is not artifactual.

Newly-hatched larva
The basal lamina underlying the limb ectoderm of a newly-hatched grasshopper is shown in Fig. 6. It is a single, continuous sheet about 90–140 nm thick. The basal lamina is attached to the ectoderm by hemidesmosomes. Beneath the basal lamina is a thick mat of fibrils. They are about 20–30 nm thick and are distinctly banded and are probably collagen fibrils.

Discussion
Our previous observations of HRP-filled pioneer neurones showed that their growth cones and associated filopodia were frequently in contact with basal lamina material underlying the limb ectoderm (Anderson & Tucker, 1988). This basal lamina material had a very different structure from the standard vertebrate basal lamina presented in reviews (e.g. Reale, 1984); it was frequently patchy, multilayered, and of variable thickness (Anderson & Tucker, 1988). In the present study we therefore examined in detail the structure of basal lamina material in different regions of the limb before, during, and after pioneer neurone outgrowth to determine if basal lamina associated with the pioneer pathway differed in structure from basal lamina elsewhere.

Embryonic basal lamina structure varies with position and developmental age
This study showed that the basal lamina underlying the ectoderm of the embryonic limb is a dynamic structure, a finding not anticipated from observation of basal lamina structure in mature systems. The basal lamina shows considerable spatial and temporal variation in continuity, thickness, granularity, and degree of layering.

In distal limb regions a basal lamina is often absent or present only as small segments (Figs 2G, 3H, 4G). In all other limb regions a continuous basal lamina is present.
Fig. 3. The basal lamina in 35% metathoracic limbs. (A) SEM of a 35% embryo. Arrowheads and curved arrows indicate locations of electron micrographs in (B) to (H). (B) Ventroanterior region of base of coxa. (C) High magnification of basal lamina from a preparation similar to (B). (D) Dorsal region of trochanter/proximal femur. (E) Posterior region of trochanter/proximal femur. (F) Ventral region of trochanter/proximal femur. (G) Dorsal region of tibia/tarsus. (H) Tip of tarsus. Bar, 0.1 mm (A), 1 μm (B,D,E,F,G,H), 0.25 μm (C).
Fig. 4. Basal lamina in 40% mesothoracic and metathoracic limbs. (A) SEM of a 40% embryo. Arrowheads and curved arrows indicate the locations of electron micrographs shown in (B) to (G). (B) Dorsal region of metathoracic coxa. (C) Ventral region of trochanter/proximal femur of metathoracic limb. (D) Dorsal region of metathoracic femur. (E) Dorsoanterior region of mesothoracic tibia. (F) Ventral mesothoracic tibia. (G) Tip of mesothoracic tarsus. Bar, 0-1 mm (A), 5 μm (B), 1 μm (C–G).
H. Anderson and R. P. Tucker

Fig. 5. Electron micrographs of 35% limb regions following ablation or removal of tips prior to fixation. (A) Stump region adjacent to the ablation. (B) Stump region 15 μm proximal to the ablation. (C) Stump region 100 μm proximal to the ablation. (D) Distal region of tip of intact control limb. (E) Distal region of an amputated tip. Bar, 0.5 μm in all cases.

The basal lamina is multilayered in several regions of the limb. This arrangement was not observed in the larva (Fig. 6). Multilayered basal lamina is generally associated with dorsal limb ectoderm (Figs 2D,F, 3D,G, 4C-F) and the base of the limb (Fig. 3B). A single layer of basal lamina is usually associated with ventral limb ectoderm (Figs 2E, 3E).

The concentration of approximately 30 nm granules within the basal lamina is greater in more proximal regions of the limb (e.g. Fig. 2A) than in more distal regions (e.g. Fig. 2H).

These regional variations in basal lamina structure do not result from fixation or staining artifacts. However, variations in electron density of basal lamina were also noted in the text, and probably arise from regional variations in the penetration of stains (Fig. 6).

Variation in embryonic basal lamina structure may reflect the normal sequence of basal lamina formation or positionally-specified differences in basal lamina formation

General agreement exists that, in vertebrates, basal lamina components are largely formed by the sheets of cells resting on the basal lamina, whereas the meshwork of collagen fibrils frequently found below the basal lamina proper is produced by fibroblasts. Insect extracellular matrix components probably have similar origins. In the case of the embryonic grasshopper limb, the basal lamina underlying the ectoderm is thought to be formed by the ectodermal cells. The collagen-like fibrils we observed beneath the basal lamina of the larval grasshopper limb ectoderm is probably contributed later by haemocytes; in Drosophila haemocytes ac-
cumulate collagen transcripts during metamorphosis (Knibiehler et al. 1987) and collect around the basal lamina surface of organs and tissues, and in the grasshopper, haemocytes bind a monoclonal antibody which binds to extracellular matrix material (Ball et al. 1987).

At the end of embryonic development the basal lamina in the limb has a structure (Fig. 6) like that commonly found in vertebrates (Reale, 1984) with a single lamina densa about 100 nm in thickness. We suggest that structural differences in the basal lamina from different proximal–distal levels of the early embryonic limb might reflect a gradient of development of the basal lamina, the limb tip showing basal lamina at its earliest stages of formation, and the limb base showing basal lamina at a more advanced stage of formation.

If this is the case, the sequence of development of the basal lamina appears to be: 1) patches of basal lamina material containing thin fibrils and small granules; 2) addition of material between patches to form a continuous sheet; 3) addition of material to form a thicker core and also addition of granules. As the limb develops it both grows in size and changes in shape. In regions where the ectoderm is dividing more rapidly and changing its curvature, the ectodermal cells may have to lose contact with their underlying basal lamina more often and secrete new layers, thus giving rise to the multiple layers of basal lamina observed in some regions of the limb.

Although no other studies have been made of developing insect basal lamina, a detailed study in the mouse (Csato & Merker, 1983; Merker, 1987) shows a similar picture: basal lamina material first appears as small vaguely demarcated plaques followed by the appearance of short segments with a typical basal lamina structure, which lengthen until the gaps close laterally. Many free basal lamina segments, branching structures, and multiple layers are also seen but these disappear after a continuous sheet is formed.

However, it is not known if limb segments are formed sequentially at the tip, or indeed if the limb grows preferentially at the tip. Therefore regional variations in basal lamina structure might result not from initiation of basal lamina formation at the tip and its progressive maturation, but from regional specification of ectodermal cells within the limb to secrete different types of basal lamina. There is considerable evidence of variability in the structure and biochemical composition of basal laminae from the same and different tissues (Grant & Leblond, 1988 and references cited therein). Comparable studies have not been made in insects. However, there is at least one potentially relevant difference among the segments of the limb - their musculature. The coxa, tibia, and tarsus (but not the femur) will form one or more specialized invaginations, called apodemes, to which is attached one end of a muscle. The other end of each muscle is attached to a particular region of ectoderm elsewhere in the limb; much of the ectoderm in the coxa and femur forms such attachment sites whereas only the proximal and distal extremes of the tibia and the proximal edge of the tarsus differentiate in this way. It has been suggested that local differences in the ectodermal basal lamina provide cues for muscle-forming cells to insert and differentiate the myoepidermal junction (Williams et al. 1984).

Variation in basal lamina structure could provide cues for guiding pioneer axons

The pioneer neurones grow through the limb in a characteristic zigzag trajectory (Fig. 1). During outgrowth their growth cones and associated filopodia are in contact with basal lamina material beneath the ectoderm (Anderson & Tucker, 1988). The present study demonstrates that the basal lamina shows distinct variations in its structure, and these are correlated with the initial direction of pioneer neurone outgrowth. Along the proximal–distal axis of the limb there is a gradient of basal lamina structure with thinner basal lamina at the tip and thicker basal lamina at the base. The initial outgrowth of the pioneers is also oriented along the proximal–distal axis, in parallel with the gradient of basal lamina structure. Around the circumference of the limb there is a tendency for basal lamina along the dorsal side to be multilayered. This is the side along which the growth cone first navigates.

How might the basal lamina influence axon outgrowth? Vertebrate neurones in culture preferentially grow over surfaces to which they adhere strongly (Letourneau, 1975). Several molecules associated with vertebrate basal lamina, such as laminin, type IV collagen, and fibronectin, can provide such adhesive surfaces (Letourneau, 1975; Carbonetto et al. 1983; Gundersen, 1985; Svoboda & O'Shea, 1987), and also vary from one region of basal lamina to another (Grant & Leblond, 1988 and references cited therein). Such variations could influence the direction of axon outgrowth.

When the pioneers reach the boundary between the future coxa and trochanter, they turn and grow ventrally along the anterior side of the limb until they contact the Cx1 guidepost cells (Fig. 1). We did not detect any abrupt difference in basal lamina structure at the trochanter–coxa boundary. There is a difference in the arrangement of the ectodermal cells in this region (Caudy & Bentley, 1987); the trochanter cells at the border are elongated and their long axes are oriented around the circumference of the limb. It is possible that the cells along the boundary and cells in the coxa secrete a basal lamina of different composition. It is known for example that glycosaminoglycans provide a poor surface for neurone outgrowth in culture (Carbo-
netto et al. 1983). The granules observed in the basal lamina of the proximal limb may be proteoglycans and could provide a barrier to neurone outgrowth into the coxa.

The molecular composition of insect basal lamina is only just beginning to be studied, and includes molecules similar to collagen IV (Blumberg et al. 1987; Cecchini et al. 1987), laminin (Fessler et al. 1987; Montell & Goodman, 1988), and fibronectin (Gratecos et al. 1988). An examination of the distribution of these and other basal lamina components within the embryonic grasshopper limb may provide useful information for evaluating the role of basal lamina molecules in directing the outgrowth of pioneer neurones.

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References


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