A study of the growth cones of developing embryonic sensory neurites

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SUMMARY

The scanning electron microscope was used to examine the growth cones of sensory neurites on the basal lamina of the trunk skin and on the myotomes in dissected embryos of the amphibian, *Xenopus laevis*. On the myotomes growth cones are large and flat with extensive lamellipodia and many filopodia. On the skin growth cones are smaller and have simpler processes particularly in more ventral positions. Where growth cones contact each other or other neurites they are very intimately apposed and show many indications of strong mutual adhesion. Fasciculation and separation of growing neurites is described and the conditions leading to fasciculation are considered. Measurements of growth cones on the myotomes and different dorsoventral regions of the skin are interpreted in terms of possible differences in the adhesiveness of these substrates. We conclude that many of our observations can be explained by differences in substrate adhesion to the growth cones but that the skin may have some special, unknown attraction for them.

INTRODUCTION

Growth cones were first described by Ramon y Cajal (1890) as 'terminal enlargements or excrescences garnished with spines'. In studies of growing neurones, both *in vitro* and *in vivo* growth cones were described as 'amoeboid' and as continually producing and retracting pseudopodia (Harrison, 1907, 1910; Lewis & Lewis, 1912; Spiedel, 1933; Hughes, 1953). More recent studies have shown that three types of process are commonly produced by growth cones:

- Filopodia (0.08 to 0.2 μm in diameter and cylindrical)
- Micropodia (branches of variable shape and diameter)
- Lamellipodia (flat, sheet-like extensions)

(Nakai & Kawasaki, 1959; Pomerat *et al.* 1965; Letourneau, 1979; Roberts, 1976; Nuttall & Wessells, 1979; Johnston & Wessells, 1980). All these processes are in intimate contact with the substrate particularly at their extremities (Luduena, 1973; Roberts, 1976; Wessells & Nuttal, 1978). By analogy with amoeboid movement in cells and protists it is assumed that these contacts are points of adhesion to the substrate and Bray (1979) has suggested that growth cones, their processes and neurites can exert tension between these points of

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attachment to the substrate. In cultured neurones the influence of substrates of different adhesiveness has also been examined, and has been found to influence the course of growth and the morphology of growth cones (Luduena, 1973; Letourneau, 1975a,b, 1979; Helfand, Smith & Wessells, 1976; Hawrot, 1980), and the degree of fasciculation (Nakai, 1960; Rutishauser, Gall & Edelman, 1978).

The structure and behaviour of growth cones have been reviewed recently by Johnston and Wessells (1980). It is clear that most information has come from studies on cultured neurones in artificial media and on artificial substrates. Our present study uses one of the few preparations where large numbers of growing neurites and growth cones can be examined in their normal environment. We have used the scanning electron microscope (S.E.M.) to examine the inside surface of trunk skin in *Xenopus* embryos. This is innervated by Rohon-Beard and extramedullary cells (Hughes, 1957; Roberts & Hayes, 1977). The way that these neurites reach the skin and some features of their behaviour on the skin are reported elsewhere (Roberts & Taylor, 1982; Taylor & Roberts, 1983). Our aim here was to examine the growth cones in different locations and on different substrates to look for possible influences on them *in vivo* that have been suggested *in vitro*.

**METHODS**

Embryos between stages 21 and 32 (Nieuwkoop & Faber, 1956) were fixed for up to 24 h in 5% glutaraldehyde in 0.05 M-cacodylate buffer at pH 7.3. After fixation embryos were washed in the same buffer for at least 5 h. Skin was then dissected from each side of the body. The skin sheets and body pieces were then dehydrated through an ethanol series and transferred to acetone for a maximum of 30 min before being critical-point dried using CO₂ in a Polaron critical-point dryer. The specimens were then mounted on aluminium stubs using double-sided Sellotape, sputter coated with gold and viewed at 10 kV in a Cambridge Stereoscan scanning electron microscope.

Growth cones were measured from projected photographic negatives. Areas were determined using a DMAC area analyser.

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**Fig. 1. Growth cones on the myotomes (my) at intermyotome clefts at stage 24.** (A) Four growth cones (1) only partly visible growing along two fasciculated neurites; (2) large flattened growth cone whose leading lamellipodia (white arrows) show high emission suggesting that they may have been torn up from their substrate during preparation. Note many short filopodia (e.g. at arrowhead). (3) Smaller branching growth cone; (4) large growth cone only loosely attached to myotome. ×3360. (B) Loosely attached growth cone in intermyotome cleft. At left is a fascicle of neurites showing processes of mutual attachment (e.g. at arrowheads). (Small white spheres are contamination.) ×6000. (C) A growth cone leaving myotome tissue (my) to contact skin (removed) which lay above plane of page. ×6000.
RESULTS

Observations of growth cone morphology have been made on dissected embryos. We have described how the sensory neurites of Rohon-Beard and extra-medullary neurons emerge from the spinal cord to reach the surface of the myotomes and then travel over the myotomes to the skin (Taylor & Roberts, 1983). The form of growth cones on the myotomes, skin and other neurites will now be considered in turn.

Growth cones on the myotomes

Here the morphology of the growth cones is at its most elaborate. They usually have many large flattened lamellipodia whose margins are closely associated with the myotome surface (Fig. 1A, fig. 1 in Roberts, 1976 and see Taylor & Roberts, 1983). The lamellipodia themselves bear large numbers of short filopodia (less than 10 μm long). Micropodia are not common. The areas of isolated growth cones are larger on the myotomes than on any other substrate (Fig. 5). The growth cones have a generally ventral orientation (where 0° is dorsal, 90° caudal, mean orientation 178°, range 110 to 300°, N = 35). A number of growth cones have been seen on the point of leaving the myotome to contact the overlying skin, which is removed during specimen preparation (Fig. 1B, C). These are not as flattened as those on the myotomes or skin and tend to extend lamellipodia in many directions. In passing between the myotomes many growth cones follow neurites or grow on top of each other (Fig. 1A and fig. 1A in Roberts (1976)). These are considered below.

Most growth cones are found on the myotomes at stages 24 and 25. After this a few were found which were somewhat smaller and lacked the extensive lamellipodia.

Growth cones on the skin

Growth cones migrate from the myotome surface onto the basal lamina on the inside of the skin. At stage 24 parts of growth cones have been found on the basal lamina near rostral intermyotome clefts. Whole growth cones are also found, some very similar to those on the myotomes (Fig. 3A and see Taylor & Roberts, 1983). Early growth cones are of variable shape with lamellipodia very closely applied to the basal lamina, micropodia and filopodia (Fig. 2A, B). On the skin...
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Fig. 2
the growth cones have a distinct ventral orientation (Roberts & Taylor, 1982).

As they grow ventrally over the basal lamina of the skin the growth cones show a gradual simplification in their morphology, and reduction in size. Dorsally, over the myotomes, they tend to have a central flattened area, some lamellipodia and more micropodia than those on the myotomes (Figs 2, 3A–F). Processes extend laterally in most directions from the sides of growth cones and can also extend from the neurite for considerable distances behind it (e.g. Fig. 2C for 25 μm). Most neurites on the basal lamina appear to be anchored by small branchlets. In the midregion of the basal lamina (ventral to lower edge of myotomes) the central flattened area and lamellipodia are rare while micropodia are more common (Fig. 4A, B; Fig. 3G–L). Filopodia often over 10 μm long extend mainly ventral and lateral to the growth cone. In more ventral regions of the basal lamina the morphology of the growth cones is still simpler. The main body of the growth cone is smaller with a few micropodia and filopodia, which can be quite long (Fig. 4C, D; Fig. 3M–T). Lamellipodia are not present in this region.

The apparent gradual changes in dimensions and morphology with dorso-ventral position were examined in more detail using photographs of 156 growth cones. Neurite diameters, the number of filopodia, and the areas of the growth cones (including micropodia and lamellipodia) were measured. Ten of the growth cones were on the myotomes where neurite diameters (mean 0.69 μm, s.d. 0.35) and the other measures (Fig. 5) were large. On the basal lamina all the measurements were less than on the myotomes. The growth cones became smaller and more simple ventrally, but fine neurites with small simple growth cones occurred in all positions on the basal lamina.

Some features of the growth cone areas need further comment. On the basal lamina overlying dorsal myotome (position 1) the areas are low (average 14 μm²) when compared to the next two more ventral positions (average 19 μm², and

Fig. 3. Tracings from photographs of growth cones to illustrate the range of form on different parts of the skin’s basal lamina. A–F dorsal, G–L mid, M–T ventral.

Fig. 4. Growth cones on the basal lamina of the skin ventral to the myotomes. (A) and (B) midregion examples with micropodia and long filopodia (small arrowheads). (C) and (D) ventral growth cones which are smaller and have few processes. In (D) the growth cone crosses a small neurite (indicated by small arrows). Magnification: ×6000 except (A) at ×4900. Stage: (A) 24, (B) 25, (C) 28, (D) 34/35.

Fig. 5. The relationship of growth cone and neurite features to their location. M are measures of growth cones on the myotomes (N = 10). Positions on the basal lamina are indicated on a dorsoventral scale of 1 to 7 (see diagram of embryo). 1 is dorsal myotome and fin. 2 is midmyotome. 3 is ventral myotome and dorsal belly. 4 to 7 are dorsal to ventral belly. Coincident points are not indicated. Open arrowheads indicate the means at each position. The solid line is based on linear regression for the positions through which it passes. (The bulk of neurites emerge at level 2 and grow toward level 7. Linear regressions were therefore calculated for data from these levels, except for data on filopodia where level 1 was also included.) The dashed line is a projection of the linear regression through positions not used in its calculation.
Fig. 4. For legend see p. 37.
Fig. 5. For legend see p. 37.
Fig. 6. (A) Dorsal growth cone at stage 26 showing complex branching pattern of processes and dorsal orientation. ×5000. (B) Small growth cone on right has just separated from neurite on left which then branches (*). One branch passes briefly under basal lamina. Ventral to myotomes at stage 28. ×5700. Note mutual attachment just before separation.

22 μm²). This results in part from the inclusion of dorsally oriented growth cones which appear in this position after stage 26, (Roberts & Taylor, 1982) when the majority of growth cones have already reached the midregion of the basal lamina, (positions 3 and 4) (Fig. 5). These extreme dorsal growth cones lack lamellipodia and have few micropodia, but produce many filopodia which are associated with small fibrils on the basal lamina. Accumulations of these fibrils are only seen near filopodia.

Growth cones on other neurites

While they make up only a small fraction of the surface over which neurites

Fig. 7. Interaction of growth cones with each other and with other neurites. (A) Two neurites (note mutual attachment process at top) whose growth cones intertwine as one branches. These are both flattened growth cones (stage 26). ×2200. (B) A small late growth cone (stage 28) crosses one neurite and extends micropodia along it (small arrows). Its micropodia contact a second neurite showing clear attachment (arrowhead). ×5758. (C) A growth cone coming from the left crosses two fine neurites extending micropodia (top one broken) along these. It then contacts a second growth cone which has just separated from the two neurites. Close mutual attachment of the growth cones is clear. Stage 28. ×5600. (D) Contact of two growth cones showing clear attachment and intertwining of a micropodium (arrow). Stage 28, ×11300.
Fig. 7
grow, other neurites are a very significant substrate as they often have strong influences on the direction of growth.

As they grow between the myotomes neurites often form bundles (Roberts, 1976; Taylor & Roberts, 1983). Compound growth cones are often found where interpretation of individual morphology is very difficult. Where one growth cone grows on another or along a neurite (Fig. 1A), contact is very intimate. Behind the active growth cones neurites make extensive mutual contact within bundles by means of small lateral flaps (Fig. 1B). These observations indicate a mutual attractiveness in both growth cones and neurites on the myotomes.

On the basal lamina neurite bundles break up and the tendency to form fascicles is weaker (Roberts & Taylor, 1982; Taylor & Roberts, 1983). The simplest interaction is when a growth cone crosses another neurite (Figs 4D, 7B, C). The probability of crossing increases as the angle of incidence approaches normal (Roberts & Taylor, 1982). In crossing, the growth cone often extends micropodia along the neurite or is slightly expanded in the region of contact. These observations and the change in direction of micropodia which contact neurites (e.g. Fig. 7B) suggest that neurites are a more attractive substrate than the basal lamina. This also applies to the surface of growth cones themselves (Fig. 7A, C, D). Examples of growth cones on each other show very close mutual contact both of the central area and of micropodia and filopodia. This is particularly clear in higher magnification pictures (Fig. 7D). In some cases, where growth cones meet, a spectacular increase in morphological complexity can occur with the production of compound intertwined micropodia and filopodia as well as processes contacting the basal lamina.

When growth cones meet neurites of other growth cones at shallower angles they fasciculate, often adopting an elongated shape with one micropodium leading growth along the contacted neurite. However filopodia are also extended onto the neighbouring basal lamina (Fig. 7C) and fasciculation is usually not maintained for long distances. Growth cones are seen separating from neurites to which, even at the point of separation, they seem to be mutually attached (Fig. 6B).

**DISCUSSION**

The growth cones on the basal lamina of *Xenopus* embryo skin and myotome tissue are similar to those of cultured *Xenopus* spinal cord neurones (Spitzer & Lamborghini, 1976) and to those of many other classes of neurones in vitro. (Johnston & Wessells, 1980). This can give confidence both in the fixation and preparative techniques for S.E.M. examination and in the relative normality of the usual types of neurone culture.

Some features of the way neurites grow are particularly clear under S.E.M. examination when compared to the more usual methods which involve sectioning. It is, for example, very easy to appreciate the general shapes of tissue blocks
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and to see the spaces between them. Once neurites have emerged from the spinal cord it is clear that they grow along surfaces (the myotomes and the skin) and are directed by the shapes of the spaces between surfaces (the intermyotome clefs and the narrow space between the skin and the underlying endoderm tissue). On a flat surface (the myotomes) or in the narrow space between flat surfaces (skin and endoderm) growth cones have a generally two-dimensional shape which is particularly clear when they are large. In more complex spaces (intermyotome cleft meets skin surface) more complex three-dimensional morphology is found in the growth cones (Fig. 1). Even at a small scale they appear to respond to the physical features of the substrate. They enter the skin via pre-existing holes in the basal lamina (Taylor & Roberts, 1983 and Fig. 7C). These observations suggest that the physical shape of the substrates over which they grow has a powerful influence on the direction of neurite growth. Until they penetrate the basal lamina, neurites follow paths of least resistance in directions of least change of direction.

Substrate effects

The changes in growth cone morphology which occur during the outgrowth of primary sensory neurones in Xenopus have been described. We will now consider whether these changes relate to different substrates for growth and whether some substrates can influence direction of growth.

In culture it has been shown that on adhesive surfaces growth cones produce more processes with more persistent contact points with the substrate. The processes include micropodia, extensive lamellipodia and numerous filopodia, which often extend for long distances over the substrate (Luduena, 1973; Letourneau, 1975, 1979). When applied to our observations these results suggest that the surface of the myotomes is strongly adhesive leading to large growth cone areas and many filopodia. Fig. 5 shows that growth cone areas and numbers of filopodia are larger on the myotomes than on adjacent regions of skin. It also shows that there is not a sharp discontinuity in neurite diameters on the two different substrates. This suggests that the larger growth cone areas and numbers of filopodia on the myotomes are not the result of a sampling error favouring large growth cones. Neurites themselves provide a substrate for growth cones but their adhesiveness cannot be assessed by the morphological features just discussed and we will return to consider this topic later. The changes in the growth cones indicate that the basal lamina of the skin is less adhesive than the myotome surface. The other flat substrate that growth cones contact is the central mass of yolky endoderm cells under the skin ventral to the myotomes. When the skin is peeled off after fixation, growth cones have never been found adhering to the surface of the endoderm. Growth cones on the basal lamina only show occasional indications of processes which had been attached to the underlying endodermal cells and were broken when the skin was removed. The endodermal surface therefore appears to be unsuitable for growth cone attachment in vivo.
**Dorsoventral position effects**

On the basal lamina of the skin growth cone size and morphology changes as one moves ventrally (Figs 3, 5). Such changes could result from changes within the neuron or be external effects, induced for example by the substrate. Since more ventral growth cones occur later in development, the changes could also relate to the age of the neurite or substrate. In considering these results it is important to note that while large growth cones with many filopodia are only present in more dorsal positions, smaller simpler growth cones are present at all locations. This means that the changes cannot be a simple function of changed substrate condition with position or of distance of the growth cone from the spinal cord. Age related changes in surface properties of fibroblasts have been reported (Aizawa, Mitsui, Kurimoto & Matsuoka, 1980). If such changes occurred in neurones and affected surface adhesiveness then age could change growth cone morphology. It would be of interest to see in vitro if growth cones showed such age related effects. Returning to the substrate, it is possible that reduced adhesiveness could occur more ventrally on the skin though one would then need to suggest that intrinsic factors made more dorsal growth cones simple. One could also suggest that, later in development, skin over the dorsal myotomes becomes less adhesive resulting in the smaller ‘late’ growth cones (Fig. 6A). However, these have other characteristics (many filopodia) which do not fit with this simple proposal. The unusual fibrils looking like filopodial branches have not been seen at earlier stages or in other positions and could be small collagen fibrils whose deposition is in some way stimulated by the growth cones. Many of the questions raised by these regional changes are amenable to experimental analysis using cultured neurones on artificial substrates or on fixed ‘dead’ skin.

An attractive hypothesis, which would be more difficult to test experimentally, is that innervated skin becomes less adhesive. This would explain why later growth cones in dorsal positions were simple but not why more ventral growth cones were on virgin skin. One would again have to propose a secondary effect like age, or distance from the neuron soma.

**Neurons as substrate**

Neurites and other growth cones provide a substrate with clear effects on the direction of growth. The S.E.M. has allowed detailed examination of the contacts made by growth cones and their processes onto neuron membrane surfaces. It is clear that these contacts are more intimate than those made with any other surface, examined in this study. This indicates that neuron surface membrane is more highly adhesive perhaps possessing special adhesion sites (Rutishauser et al. 1978). This conclusion applies to all parts of the growing neurite, its growth cone and the lamellipodia, micropodia and filopodia. No indications of any repulsive effects such as those proposed by Dunn (1971, 1973) have been noticed. Neurites and growth cones therefore provide small areas of highly
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Most neurites reach the surface of the myotomes in bundles by fasciculating with earlier pioneers (Taylor & Roberts, 1983). The formation of bundles of neurites is easy to understand in view of their mutual attractiveness. These bundles begin to separate on the myotomes and this process continues once they reach the skin. The large areas of less adhesive surface provided by the myotomes and skin can compete with the small areas of high adhesion offered by neurites. The fasciculation tendency seems strongest in the intermyotome cleft where neurites are at their highest density (offering a large adhesive surface) and are also at their most highly oriented (all running parallel). Once separation begins, density and orientation will decrease encouraging further separation. On the basal lamina and on the myotomes growth cones can fasciculate with and separate from other neurites which consequently influence their direction of growth (Roberts & Taylor, 1982). All these phenomena can reasonably be explained in terms of differential adhesion between the substrates encountered by the growth cones (Letourneau, 1975a, b; Helfand et al. 1976).

CONCLUSION

We have paid particular attention in the present study to the roles that features of the substrate shape and adhesiveness play in directing growth cone extension. If our interpretation is correct the myotome surface is more adhesive than the basal lamina of the skin. The sensory neurites normally grow from the myotome surface onto the basal lamina, from a more to a less adhesive surface. Neurites on the myotome also do not seem to grow into intercellular clefts or holes between myotome cells, whereas they do penetrate such holes between skin cells. Taken together these observations suggest that the skin, which will ultimately be innervated by these neurites, exerts some unknown attractive influence on their growth cones so that they leave the myotome surface and grow through the basal lamina to their target cells.

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