J. Embryol. exp. Morph. Vol. 70, pp. 215–224, 1982
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The development of the peripheral trigeminal innervation in *Xenopus* embryos

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SUMMARY

The development of the ophthalmic, maxillary and mandibular nerves has been followed in *Xenopus laevis* embryos from the first emergence of growth cones from the trigeminal ganglia until the establishment of functional innervation of the skin or cement gland. The course of each main nerve is highly predictable and follows pre-existing openings between blocks of other tissues. The development of the mandibulary nerve was observed most easily. Like that of the other trigeminal nerves it falls into three stages: (1) A pioneer neurite emerges and a nerve forms as other, later neurites fasciculate with this. (2) On reaching the inside surface of the cement gland the neurites separate and penetrate holes in the basal lamina. (3) The neurites grow between the cells they will innervate and form free nerve endings. The scanning EM observations have been confirmed by electrical recordings from trigeminal neurones. The role of pioneer fibres and substrate guidance are discussed.

INTRODUCTION

If we are to discover what factors determine the course of nerves which innervate peripheral tissues, we must first of all have an accurate description of the way in which particular nerves develop. This requires an anatomical technique which can resolve the fine pioneer neurites found during early development. The scanning electron microscope (SEM) allows more than adequate resolution and we have used it to examine the course of developing neurities as they grow over the tissue blocks of young embryos. Our aim in the present study was to follow the first innervation of the head skin and cement gland in embryos of *Xenopus laevis* using the SEM and to check the development of function by making electrophysiological recordings from young embryos.

We hoped to evaluate the role of pioneer neurites in the formation of the main trigeminal nerves. A special role for pioneer neurites was first suggested by Harrison (1910). He proposed that early neurites could find correct pathways by trial and error. Once this was done, and providing that wrong connexions were then eliminated, later fibres could then follow the pioneers to their correct terminations (Weiss, 1950).

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In earlier work on *Xenopus laevis* Roberts and Blight (1975) showed the cement gland to be innervated by trigeminal neurons which respond to mechanical stimulation with a discharge which inhibits swimming. It was later shown that the rest of the head skin was innervated by two distinct classes of trigeminal receptor neurones; movement detectors which inhibit swimming like the cement gland receptors, and rapid transient detectors which have a more phasic discharge and have excitatory effects on behaviour (Roberts, 1980).

The trigeminal complex has been studied anatomically by Coghill (1916) in *Amblystoma*, and by Hamburger (1961) in the chick. The complex contains three ganglia producing three separate roots; the ophthalmic and maxillary ganglia which are derived from the Gasserian epidermal placode, and the mandibulary ganglion which forms from a collection of neural crest cells that lie below the other two ganglia (Knouff, 1927 on *Rana*, and Stone, 1924 on *Amblystoma*).

MATERIALS AND METHODS

Embryos were obtained from breeding pairs of adult *Xenopus laevis* by priming and injecting with chorionic gonadotrophin; they were allowed to develop in aerated tap water and staged according to the normal tables of Nieuwkoop & Faber (1956).

Electron microscopy

Embryos at stages 21–34 had their egg membranes removed while still in tap water, before fixing in 5% glutaraldehyde in 0·05 molar cacodylate buffer at pH 7·3 for 2 h. They were transfered to buffer and dissected under the binocular microscope using etched and mounted tungsten needles. Body and skin pieces were dehydrated through a series of alcohols, then transferred to acetone and dried using CO_2 in a Polaron or Tousoumis critical-point drier. The specimens were then sputter coated with gold, and viewed in a Cambridge Stereoscan S4 microscope.

Electrophysiology

Electrophysiological recordings were made from curarised animals pinned out on a rotatable rubber table in Ringer's solution of the following composition: NaCl 100 mM, KCl 2·5 mM, $NaHCO_3$ 20 mM, NaH_2PO_4 0·1 mM, $CaCl_2$ 1·8 mM (pH 7·6).

Suction electrodes with tip-opening diameters of between 30 and 120 μm were prepared as described in Roberts (1980), and placed on the trigeminal ganglion after removing small areas of overlying skin with fine mounted needles, incising posterior to the trigeminal ganglion so as to leave as much of the head skin as possible intact. Suction was applied using a syringe driven by a micrometer attachment, so as to minimise damage to the cells of the trigeminal ganglion during experiments.

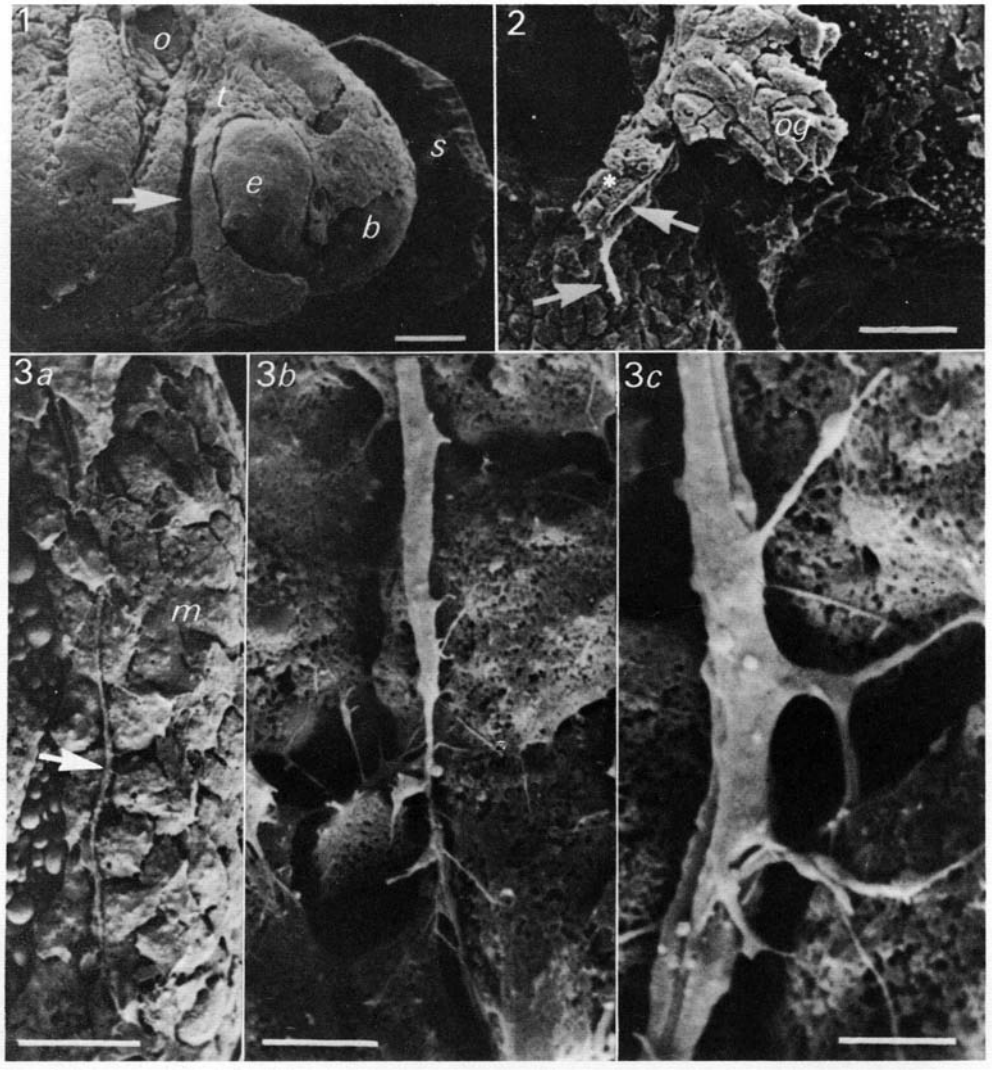

Fig. 1. Stage 25. Head with skin removed showing general topography of the area viewed from the right side. For full description see text. Scale bar = 100 μm.

Fig. 2. Stage 24. Extensive dissection with eye and connective tissue removed to reveal the trigeminal complex attached to the hind brain. Orientation and view as in Fig. 1. At right is the ophthalmic ganglion (*og*), and more ventrally the maxillary ganglion (*) which overlies the mandibular ganglion. Below this is the broken stub of the mandibular nerve (lower arrow), while growing down the near side is the less well developed maxillary nerve (upper arrow). Scale bar = 50 μm.

Fig. 3*a*. Stage 24. Bundle of mandibular neurones (arrow) growing down the rear surface of the anterior presumptive jaw muscle segment (*m*) on the right side of the animal from the trigeminal complex (*t*) at the top towards the cement gland off the bottom of the picture. Scale bar = 25 μm. 3*b*. Naked pioneer fibre at the tip of bundle shown in Fig. 3*a*. Scale bar = 5 μm. 3*c*. Following growth cone showing the process of fasiculation forming the bundle shown in Fig. 3*a*. Scale bar = 5 μm.

Receptive fields were plotted by stimulating the head skin locally using a mounted gerbil hair, either hand held or driven manually by a micromanipulator. The stimulus response was monitored on a storage oscilloscope and an audiomonitor. Drawings of the head were made using a camera lucida, and the receptive fields for hand-held stimulation plotted by referring to features of the head skin, such as the eye, cement gland and pigment cells, where present, to estimate the position of the stimulus. The stimulus from hand-held probes consisted of short strokes. Where a micromanipulator probe was used, the point of stimulation was marked directly on the drawing using the camera lucida; the form of stimulation being a gentle poke approximately normal to the skin surface.

Results were stored on magnetic tape for later analysis and filming.

RESULTS

Electron microscopy

The general topography of the head area is shown in Fig. 1 where the skin has been removed, leaving a loose section rostrally (*s*), and revealing the brain (*b*) and the eye vesicle (*e*). The hollow (*o*) was previously occupied by the otic vesicle. Just anterior to this is a groove between two sections of presumptive

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Fig. 4. Stage 24. Stellate cell from the trigeminal complex ensheathing the bundle of mandibular neurones. Scale bar = 2·5 μm.

Fig. 5. Stage 28. Inside surface of the cement gland showing growth cones (arrowheads) and neurites entering holes between the secretory cells (arrows). The secretory cells are also seen in longitudinal section at extreme right (*sc*). Scale bar = 20 μm.

Fig. 6. Stage 26. Longitudinal section through cement gland showing a growth cone between the parallel secretory cells, heading towards the secretory surface at bottom right corner out of the picture. Scale bar = 2 μm.

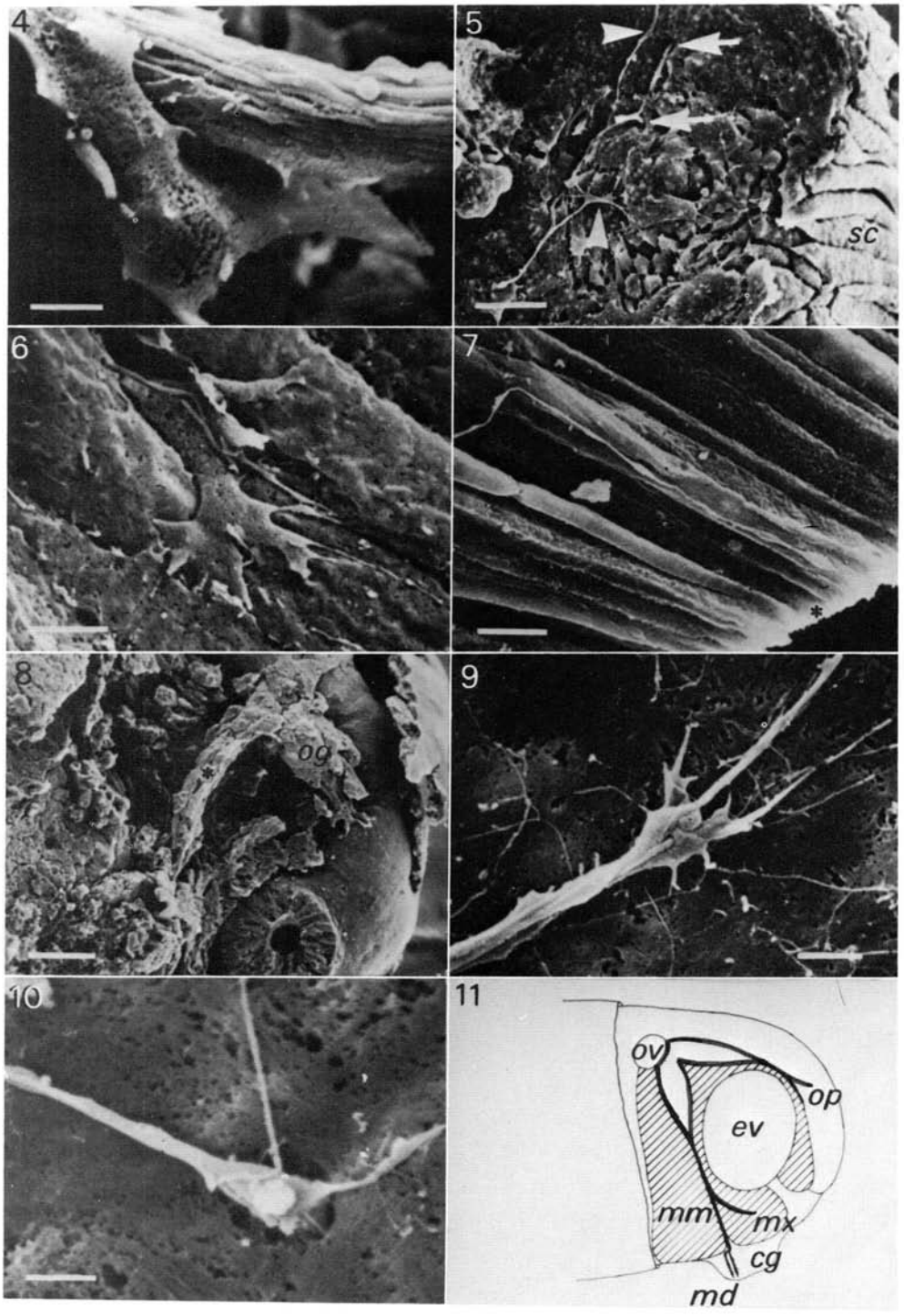
Fig. 7. Stage 33/34. Section through cement gland showing single neurite lying between the narrow secretory cells and terminating just below the secretory surface (*) shown at bottom right. The proximal end of the neurone has been displaced by the dissection. Scale bar = 10 μm.

Fig. 8. Stage 24. Similar dissection to Fig. 2. The maxillary/mandibular ganglia (*) have now extended ventrally and processes from the ophthalmic ganglion (*og*) are now visible growing rostrally on the brain surface just above the position of the eye stalk. Scale bar = 50 μm.

Fig. 9. Stage 28. Composite growth cone on the inside surface of the head skin just above the eye. Scale bar = 2·5 μm.

Fig. 10. Stage 26. Final form of innervation in the head skin showing a neurone entering a hole in the basal lamina. Scale bar = 2 μm.

Fig. 11. Diagram of animal in late twenties stage showing the positions of the eye vesicle (*ev*), cement gland (*cg*), otic vesicle (*ov*), presumptive jaw muscle masses (*mm*), and the paths of the main branches of the mandibular nerve (*md*), maxillary nerve (*mx*) and the ophthalmic nerve (*op*).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jaw muscle which runs from the position of the trigeminal complex (*t*) (hidden by other tissue) ventrally to the position of the cement gland (removed in this specimen). This groove (indicated by the arrow) is already present by stage 22, before any nervous outgrowth, and forms a channel down which the mandibulary and maxillary nerves grow.

The mandibular, maxillary and ophthalmic ganglia form a discrete mass which can be left attached to the hind brain after dissection as in Fig. 2. The right-hand lobe is the opthalmic ganglion (*og*), while the more ventral lobe (*) is formed by the mandibular ganglion and the maxillary ganglion which overlies it. This more ventral lobe projects into the top of the groove and directs any neurones emerging from it into that groove. The mandibular nerve is the first to appear around stage 22/23 when it can be seen on the mesial surface of the ganglion cells. The maxillary nerve emerges on the lateral surface at a slightly later stage (see Fig. 2).

The mandibular nerve subsequently transfers to the rear surface of the muscle primordium that forms the anterior edge of the groove (Fig. 3*a*). The path of the nerve is determined by a single naked pioneer neuron (Fig. 3*b*), whose neurite is then rigourously followed by later developing neurons (Fig. 3*c*), thus forming a tightly packed fascicle. At some later stage this bundle becomes ensheathed by cells from the trigeminal mass which reach their positions by migrating down the neurons themselves (Fig. 4).

The mandibular nerve emerges from the bottom of this groove around stage 25–26, still as a discrete bundle, but then splits up when it meets the almost perpendicular surface presented by the inside of the cement gland. Growth cones are then found singly or in small groups on this surface and some are seen entering holes between the secretory cells (Fig. 5).

Longitudinal sectioning of the cement gland at stage 26 shows the growth cones of these neurites between the narrow secretory cells (Fig. 6), and later on (around stage 33/34) the final pattern of innervation by a neurite with periodic varicosities and a naked nerve ending just below the secretory surface (Fig. 7).

The maxillary nerve which grows from the lateral surface of the trigeminal complex (Fig. 2) appears less well developed in the early stages and is therefore assumed to arise slightly later than the mandibulary nerve, though still around stage 22/23. Like the mandibulary nerve it transfers to the anterior surface of the groove, but then it bends forwards, as if following the contour of the optic stalk that supports the eye, so that eventually it innervates an area of skin anterior to that of the mandibular nerve (see Fig. 11).

The ophthalmic ganglion has a more anterior/posterior axis (see Fig. 2) and neurons emerging from this ganglion are therefore directed in a more horizontal direction. Those growing forwards are directed into a channel that is formed between the brain surface and the crescent of connective tissue that surrounds the eye. The neurones start off following this channel, adhering to the brain surface (Fig. 8), but their path cannot be traced further because they become

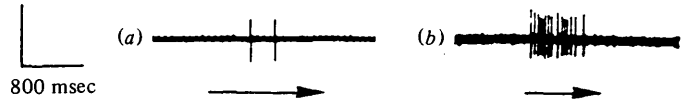

Fig. 12. Typical response of rapid transient detector (*a*) and movement detector (*b*) to a short stroke with a hand held mounted gerbil hair (arrows). The two classes of receptor are clearly distinguishable; movement detectors have a tonic discharge of many impulses, and rapid transient detectors a more phasic discharge with only one to three impulses per stroke. Voltage calibration: (*a*) 0·3 mV/cm, (*b*) 0·5 mV/cm.

covered by connective tissue. However, by stage 26 single neurites can be seen emerging through this connective tissue, and also growing on the inside surface of the skin (Fig. 9), before entering holes in the basal lamina (Fig. 10).

Since the growth cones from these three ganglia cannot be distinguished once they have reached the skin it has not been possible with this technique to determine the extent of innervation by each bundle, or to say whether the mandibular nerve innervates areas other than the cement gland. Figure 11, however, illustrates the main features of the head and the paths of the main nerve bundles from the trigeminal ganglion.

Electrophysiology

Movement detector and rapid transient detector receptive fields were identified in the head skin using their characteristic stimulus responses (Fig. 12, and see Roberts, 1980).

Movement detector receptive fields were found near the cement gland as early as stage 25, but more commonly at stage 26 (Fig. 13*a*). Movement detectors were found in the skin over the anterior eye and front of the head slightly later in stage 26 (Fig. 13*b*). By stage 26 the cement gland itself is innervated. In succeeding stages movement detector receptive fields appear over an extending area and if an individual receptive field is followed through its early development, it's size can be seen to increase as shown in Fig. 13*c*. By stage 32 the entire head skin is innervated by movement detectors, and no differences in the receptive fields or their properties were noted in later stages up to stage 36.

The development of rapid transient detectors is not as straightforward to follow as that of movement detectors. They were usually first found over the eye at stage 26 or 27 (Fig. 13*d*), and by stage 33 could be detected over the whole head surface (Fig. 13*e*). In general it was not possible to determine accurate receptive fields because of their unreliable response to repeated stimulation (Roberts, 1980).

DISCUSSION

We have followed the development of the three major trigeminal components (ophthalmic, mandibular and maxillary) and in each case development can be divided into three phases: (1) Neurites emerge from the ganglion and fasciculate

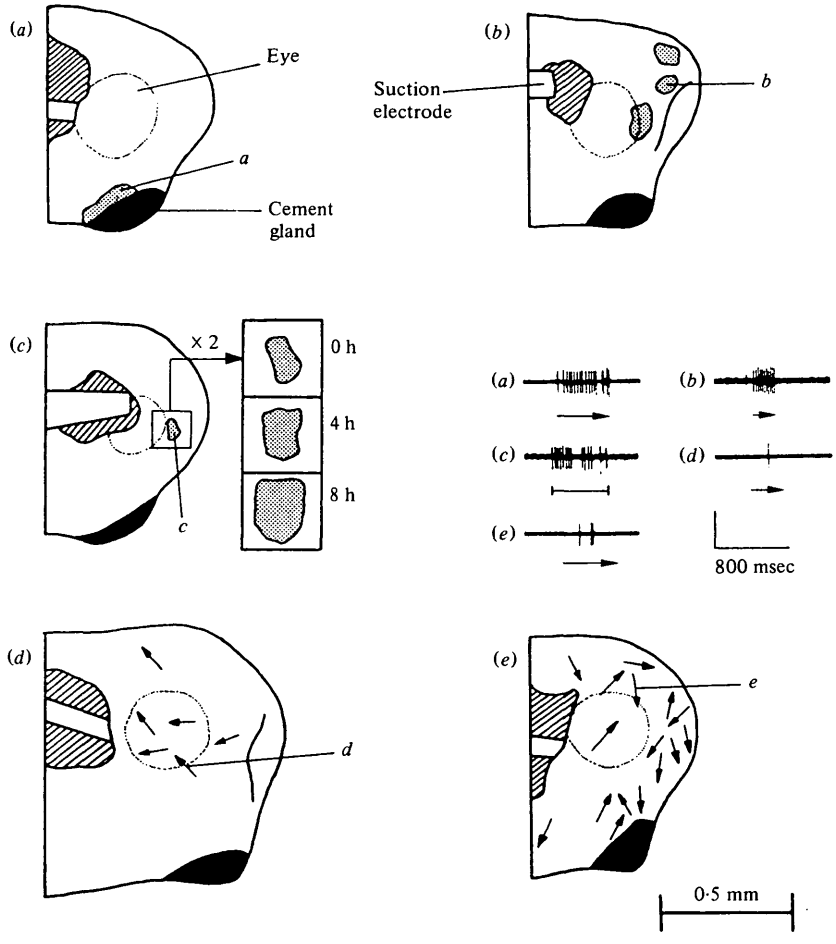

Fig. 13. Receptive fields and areas for movement and rapid transient detectors. Hatched area indicates skin damaged during placement of the suction electrode. (*a*) Movement detector receptive field (stippled) above the cement gland at late stage 25. Record shows typical response to a stroke across the field with a hand held mounted gerbil hair. Arrow indicates duration of stimulus. (*b*) Three movement detector receptive fields in a stage-27 embryo. These were the only areas of innervation found in the region of head skin extending from the eye to the dorsal and frontal midline. Record shows the response to a short stroke. (*c*) This shows the growth of a movement detector receptive field in a stage-26 embryo over the course of eight hours. The growth was approximately radial, though with initial elongation along the dorsoventral axis. The field was plotted using a micromanipulator driven probe, giving small indentations of the skin. A typical response is shown, the bar indicates the stimulus duration. (*d*) Rapid transient detectors in a stage-27 embryo. The arrows indicate the direction and length of the strokes which evoked a response, and the discharge from one of these strokes is shown. (*e*) Stage-33 embryo showing rapid transient detector responses over most of the head skin. The trace shows the discharge to a single stroke as indicated by the arrow. Voltage calibration: (*a*) 0·8 mV (*b*) 0·6 mV (*c*) 0·4 mV (*d*) 0·8 mV (*e*) 0·8 mV.

with each other for some distance to form a nerve bundle before they reach the skin. (2) On the basal lamina, neurites separate before penetrating into the skin. (3) In the target tissue they form free nerve endings. The electrophysiological recordings, from trigeminal ganglion cells, are compatible with the SEM observations both in the locations of first innervation and in its timing. In particular they show that innervation does not occur during the first phase of the initial outgrowth and formation of the nerve.

The mandibular nerve has been the most straightforward to follow from the appearance of a single pioneer neurite at the ganglion, to the establishment of typical swollen nerve endings between the cells of the cement gland (Roberts & Blight, 1975). Outgrowth of mandibular neurites is not synchronous; the first neurite to emerge we call the pioneer neurite and all later neurites follow its course by fasciculating with it to form the mandibular nerve. This process occurs on the surface of presumptive jaw muscle and before the nerve becomes wrapped in sheath cells. This strong tendency to fasciculate contrasts with the behaviour of Rohon–Beard cell neurites which innervate trunk skin and only fasciculate for short periods forming rather indistinct nerves (Taylor & Roberts, 1982). In trunk and head neurite bundles separate on reaching the basal lamina of the skin and individual neurites grow through holes in the basal lamina to their sites of innervation. In contrast to the trunk (Roberts & Taylor, 1982) sensory function by the movement detector neurones of the trigeminal seems to be established quickly (within one developmental stage of one to two hours).

Once the pioneer has grown out from the ganglion the course of the mandibular nerve to the cement gland could be entirely directed by fasciculation with the pioneer (Weiss, 1950). The process seems far from random. There is no evidence that a single pioneer fibre grows out, takes wrong turnings, and is only later followed by the remaining neurites once its own course and termination have been corrected (cf. Goodman & Spitzer, 1979). The pioneer grows down a preformed groove and is a pioneer simply in being the first neurite. Whether the groove acts as a mechanical guidance or 'substrate pathway' (Katz & Lasek, 1979) remains open to question. So does the problem of the break up of the nerve on reaching the cement gland. However, now that we have an accurate description of the normal events of development at the single neurite level, we can hope to devise experiments that may eliminate some of the many possible influences on the direction of growth of these neurites.

We would like to thank Bob Porter and Steve Martin for technical assistance and the MRC for financial support.

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(Received 14 December 1981, revised 7 March 1982)