Development 103, 97-110 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

Specific guidance of motor axons to duplicated muscles in the developing amniote limb

R. VICTORIA STIRLING and DENNIS SUMMERBELL*

The National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK

* For reprints

Summary

The effect of alteration of limb pattern upon motor axon guidance has been investigated in chick embryos. Following grafting of the zone of polarizing activity (ZPA) into the anterior margin of the early limb bud, limbs develop with forearms duplicated about the anteroposterior axis. The position of motoneurones innervating the duplicated posterior forearm extensor EMU was mapped by retrograde transport of horse radish peroxidase (HRP). The motor pool labelled from injection into the anteriorly duplicated EMU muscle is consistently similar to that supplying the posterior EMU muscle on the unoperated side of the embryo. In those cases where the axons are well filled, their trajectories from the injection site are observed to change position within the radial nerve to specifically innervate the duplicated muscle. The axons modify their trajectories proximal to the level of limb duplication in a region where there is no change in the pattern of overt differentiation of the limb cells. This suggests that axons may use a cell's positional value to navigate and provides significant support for the theory of positional information.

Key words: motoneurone, neurone, innervation, ZPA, zone of polarizing activity, pattern formation, positional information, chick embryo, limb.

Introduction

We are interested in how motor axons reach their appropriate target muscles during development. Experiments in which the spatial relationship between the spinal cord and the limb are altered prior to axon outgrowth in the chick embryo suggest that there is a topographic projection from cord to limb. Thus, following anteroposterior reversal of the limb bud (Stirling & Summerbell, 1985) or craniocaudal reversal of the spinal cord (Lance-Jones & Landmesser, 1980), the trajectories of some motor axons are changed to compensate for the reversal. The local environment of the growing axons seems to control the route axons take (Tosney & Landmesser, 1985*a*,*b*; Lewis, 1978; Stirling & Summerbell, 1987), providing that the axons are invading 'familiar' territory (Stirling & Summerbell, 1983). When the territory is unfamiliar, e.g. dorsal nerves invading ventral limb, the axons innervate muscles by passive deployment (Summerbell & Stirling, 1981). The failure of motor axons to reach appropriate targets following reversal of more than four segments of spinal cord (Lance-Jones & Landmesser, 1980) or dorsoventral reversal of the limb (Summerbell & Stirling, 1981) has led to the current view that axons are guided by local rather than long-range chemotactic guidance cues (Stirling & Summerbell, 1987; Tosney & Landmesser, 1985*a*,*b*). The axons do not become matched to myoblasts from the same segmental level as they pass through a somite, nor do the axons follow the path traced by myoblasts from an equivalent segmental level as they precede them into the limb. The pattern of motor innervation following reversal, rotation or exchange of somites in embryos before axons have left the cord is normal (Keynes *et al.* 1987).

The cues guiding the motor axons, therefore, must reside in the processes that determine the pattern of the limb itself. This paper describes the innervation of muscles in limbs whose pattern has been altered in a specific fashion. When cells from the posterior lateral margin of the early limb bud are grafted to the anterior margin of a host limb bud, a supernumerary limb develops which has mirror-image symmetry to the original limb (Saunders & Gasseling, 1968; Tickle *et al.* 1975). In such limbs, the posterior muscles of the

forelimb are duplicated in an anterior position. This situation provides a special opportunity to investigate motor axon guidance to targets that are not simply displaced as in AP reversals. It provides information about both the control of pattern formation in the developing limb bud and also the subsequent innervation of the limb.

Following a ZPA graft, cells that would normally have formed anterior extensor muscles and which would have been innervated by a cranial motor pool are now going to form a muscle that anatomically resembles a posterior extensor muscle which would normally be innervated by a caudal brachial motor pool. Here we describe the innervation of the duplicated posterior forearm muscle, extensor metacarpi ulnaris (EMU) in such duplicated limbs. We chose this particular muscle rather than those we have used in previous papers for several reasons. First, it is reliably duplicated with clear separation between the two muscles. Second, the motor pool for EMU is spatially well separated in the cord from the motor pool for the anterior forearm extensor EMR. Third, in the upper arm the triceps and biceps muscles are primarily dorsal and ventral muscles rather than posterior and anterior muscles. Fourth, we did not use the forearm flexor carpi ulnaris because it has no obvious anterior counterpart.

In the normal limb, the motor pool to the anterior forearm extensor (EMR) is situated in the lateral motor horn in the rostral 14th spinal cord segment while the pool for the posterior forearm extensor muscle (EMU) is found in the caudal 16th segment. The separation of the pools to these two muscles make this an ideal situation to test the effect of changing the limb pattern on motor axon guidance. Will the motoneurones that normally innervate the posterior (EMUp) muscle also innervate its anterior duplicate (EMUa) or will the duplicated muscle be innervated by cells that normally supply the anterior EMR muscle? There is no comparable arrangement for the forearm flexor muscles. This is probably part of the reason why the innervation of duplicated forearm flexor muscles is so variable yielding no clear answer to the question of specificity (Neset *et al.* 1985; and our own unpublished observations).

Our results show that the anterior duplicated extensor muscle (EMUa) is innervated by motoneurones located in the same position in the spinal cord as those supplying the muscle on the unoperated side (EMUc). Some motor axons from this caudal pool reach the posterior muscle as in normal animals while other axons from the same pool selectively innervate the anterior duplicated muscle by changing their position throughout their path along the radial nerve. We also observed that this selective movement of axons occurred proximal to the overt level of duplication of limb pattern. Our results suggest that axons respond directly to the signal from the ZPA or indirectly to changes in cellular positional value specified by the ZPA. Axonal navigation does not depend on overt differentiation of the embryonic field. This has implications for theories of both positional information and nerve guidance.

Materials and methods

Grafting

Fertilized chicken eggs from a mixed flock (Little Sussex and Rhode Island Red, Needle Farm, Enfield) were incubated in a humidified incubator on stationary shelves at 38°C. Eggs were windowed and staged (according to Hamburger & Hamilton, 1951) on the 4th day of development then returned to the incubator until needed for operating. Host embryos were selected at either stage 17 or stages 18–19 (see Summerbell & Hornbruch, 1981, for general operating techniques). A small piece of tissue about 200×200 μm was removed from the anterior border of the right wing bud by cutting through the thickness of the limb (see Fig. 1). Donor buds were selected at stages 20–22 and an equivalent-sized piece of tissue was removed from the posterior lateral edge of left or right wing buds (see maps of ZPA activity, Honig & Summerbell, 1985), transfixed with a platinum pin and transferred to the host egg. The graft was pinned into the prepared graft site with the pin then the host embryo was sealed with Sellotape and returned to the incubator.

Labelling

At 9 days of incubation (stage 33–35), we selected the embryos with good duplicated limbs. We required that stage-17 host embryos should be reduplicated from the shoulder, so that the humerus lay at right angles to the spinal column with good mirror-image symmetry of the whole limb having the digit pattern **4334**, **43234** or **432234** (Tickle *et al.* 1975). In later hosts, we required that the limb should be reduplicated at least from the level of the elbow so that there was clearly a duplicate ulna at the anterior margin and a similar digit pattern to those selected from stage-17 hosts (Fig. 1C). The selected embryos were eviscerated and the spinal cord exposed by ventral laminectomy in oxygenated Tyrode's solution. Cases in which it was obvious that the laminectomy had damaged the spinal roots were discarded. For retrograde mapping, muscles were injected with 40% HRP (Boehringer type II) and specimens left for 2 h at 37°C followed by 5 h at room temperature in the oxygenated Tyrode's solution. On the unoperated (control) side, we injected extensor metacarpi ulnaris (EMUc) or extensor metacarpi radialis (EMRc); on the operated side, we injected the posterior extensor metacarpi ulnaris (EMUp) or its anterior duplicate (EMUa) or both. For orthograde axon mapping, small injections of HRP were made into selected segments of ventral spinal cord or into single dorsal root ganglia (DRG). Following axonal filling, the limbs were removed from the trunk. The spinal column was fixed in cold 2%

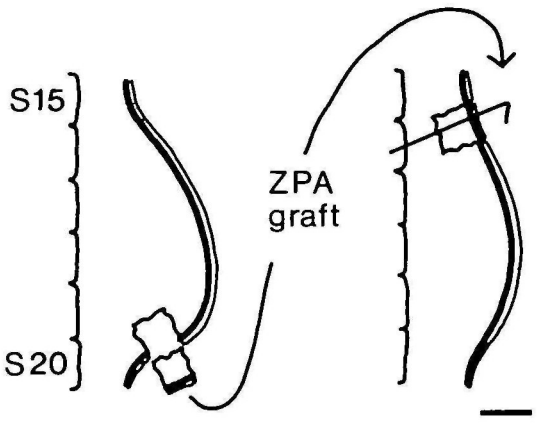
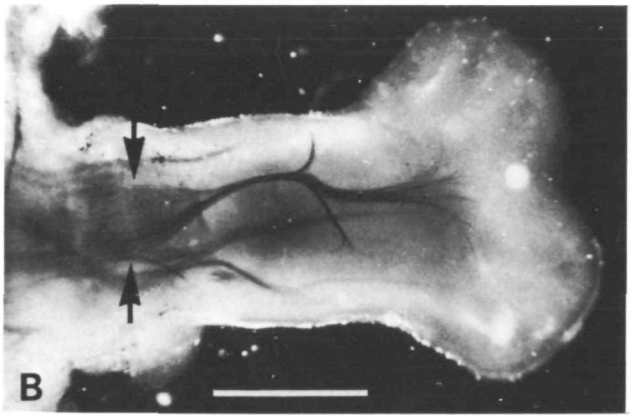
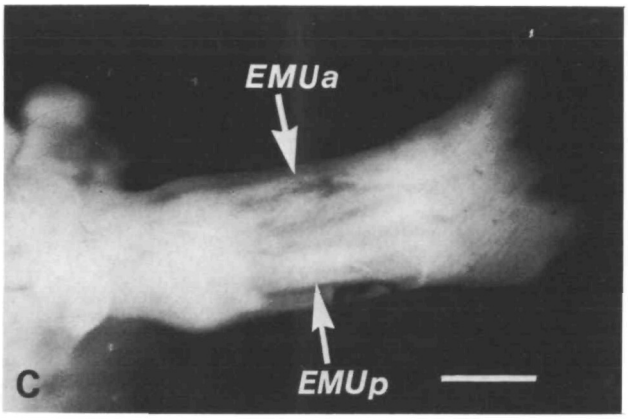


Fig. 1. (A) Diagram showing the transfer of a ZPA graft from a stage-19 donor embryo (to the left) into the anterior margin of a stage-18 host (to the right). S15 marks position of 15th somite, S20, that of 20th somite. Bar, 200 μm. (B) Dorsal view of a whole mount of a duplicated wing at stage 29 stained with the modified Bodian silver method, host axis anterior upwards. Note how the central radial nerve is spread out laterally (between arrow points) over the humerus, beyond the elbow it sends symmetrical branches to posterior and duplicated tissues. Bar, 1 mm. (C) Dorsal view of a duplicated limb just after HRP injection into the EMUa muscle, host axes anterior upwards. The HRP in the injected muscle is a diffuse light brown (grey in photograph). The darker patch at the posterior edge is blood from the posterior marginal vein. *EMUp*, extensor metacarpi ulnaris (original posterior muscle); *EMUa*, extensor metacarpi ulnaris (anterior duplicate muscle). Bar, 1 mm.





glutaraldehyde in 0·1 M-phosphate buffer at pH 7·2 for 2 h and left in buffer with 20 % sucrose overnight. The specimens were embedded in gelatine albumin, sectioned transversely at 60 μm on a freezing microtome and stained with cobalt/nickel-intensified DAB as described previously (Summerbell & Stirling, 1981). The limbs were fixed in 2 % glutaraldehyde in an extended position overnight before extensive washing and staining as whole mounts with DAB (Landmesser, 1978). The limbs were dehydrated through Langs alcohols before embedding in paraffin wax and sectioning transversely at 10 μm. Sections were counterstained lightly with cresyl violet.

Reconstructions

The pattern of the limb nerves and positions of labelled fibres were reconstructed (Stirling & Summerbell, 1985) from *camera-lucida* drawings of serial sections using a graphics pad digitizer and an interactive serial section reconstruction program (Shepherd *et al.* 1984). We digitized sections 200 μm apart for the limb, 100 μm apart for the nerves and 120 μm apart for the spinal cord. Three-dimensional viewing of the reconstructions using red and green anaglyphs makes it possible to stack the sections in their true orientation with each other which is essential for accurate analysis. Without the three-dimensional image, the full reconstruction is difficult to interpret, so for our illustrations (see below) we have chosen sections from the full reconstructions that give sufficient information for the reader to follow the relative positions of the axons within the nerves from two-dimensional diagrams.

Bodian staining

A few limbs were stained using the Lewis modification of the Bodian method, to show the overall nerve pattern (Lewis, 1978).

Results

Positions of motoneurones innervating EMUa

There were 94 survivors from 144 operations, in 52 of these both the anatomy and laminectomy were good and we injected the anterior duplicated EMUa muscle on the operated side and EMUc or EMRc on the unoperated side. Operated side motor pools were well labelled in 37 animals. The pool position of the EMUa muscle in the great majority (34) was characteristic of the normal pool for the posterior EMUc muscle in unoperated limbs (Figs 2, 3A,B) i.e. lateral motoneurones within the lateral motor column in segments 15 and 16 were labelled. Usually there were fewer cells labelled on the operated side, often this meant that there were no labelled cells at the level of the 15th spinal root (Fig. 2). In just three animals, the labelled cells were also lateral, but occupied segments 14 and 15, a position characteristic for EMR injection in unoperated wings (Fig. 3).

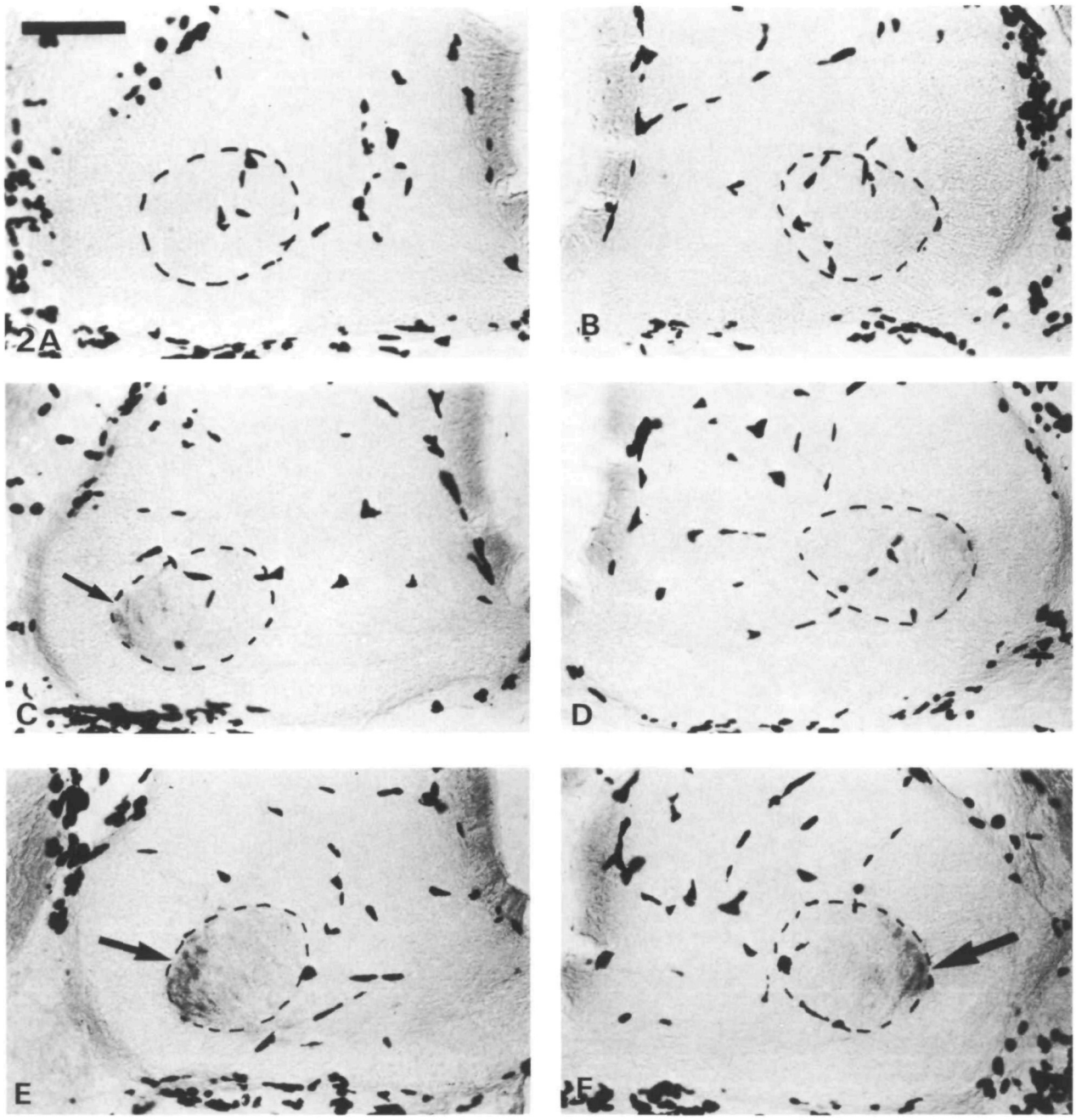


Fig. 2. Photomicrographs showing the position of HRP-labelled motoneurones in the motor horn (outlined with dashed lines) at the level of the 14th (A,B), 15th (C,D) and 16th (E,F) spinal nerves. On the unoperated side (A,C,E) EMUc was injected with HRP; on the operated side (B,D,F) its anterior duplicate EMUa was injected. Arrows show position of labelled cells. On the unoperated side there are a few labelled cells at spinal nerve 15 (C, small arrow), the main pools are at spinal nerve 16 (E,F, large arrows). EMUc, extensor metacarpi ulnaris (control). Bar, 100 μm.

Pattern of nerves and axon trajectories for EMUa muscle injection

While it is easy to obtain good trajectories from orthograde spinal cord injections, retrograde fills reliably label the motoneurones without filling the axons all the way to the plexus. Figs 4 and 5 are reconstructions of unoperated and operated limbs in one of the six cases where axons filled well enough to follow their trajectories from the injection site through the plexus. The micrographs (Fig. 6) show the position of the filled axons at selected positions in the two limbs. On the unoperated side (Fig. 4), the filled axons are situated dorsally in the 15th and 16th segmental nerves and maintain their posterior position within the dorsal radial nerve. Notice that triceps axons occupy the caudal unlabelled sector of

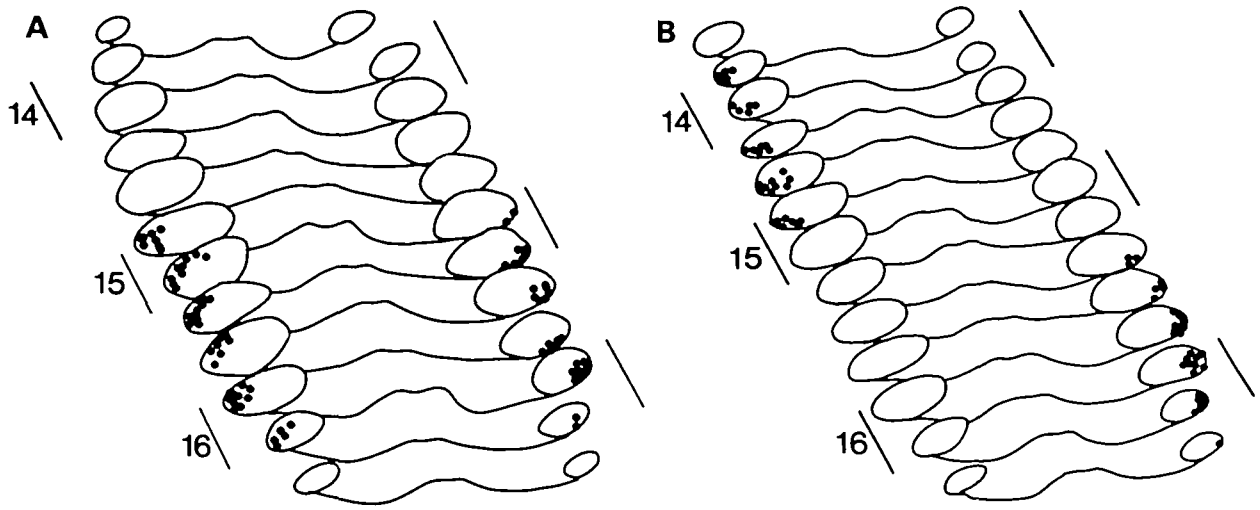


Fig. 3. Computer-generated reconstructions from camera-lucida drawings of serial sections every 120 μm through the brachial motor horn showing the position of labelled motoneurones on unoperated (to the left) and operated (to the right) sides in two animals with duplicated limbs. The origin of the 14th, 15th and 16th spinal nerves is indicated by the bars on either side. (A) On the unoperated side, EMUc was injected; on the operated side EMUa. The position of labelled pools is symmetrical. (B) On the unoperated side, the anterior extensor EMR was injected; on the operated side EMUa. Despite the anterior position of both injected muscles, the pools are asymmetrical; the pool position on the operated side being characteristic for EMUc muscle injection.

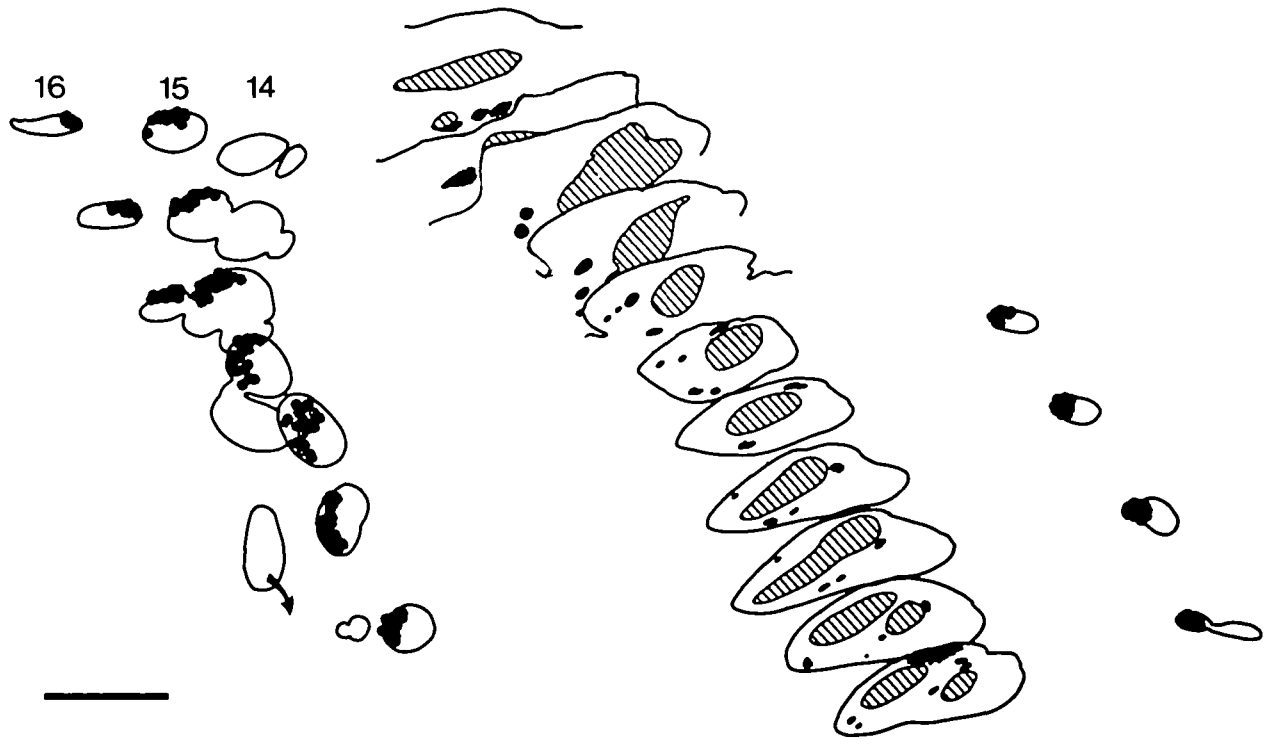


Fig. 4. Computer-generated reconstruction from serial sections (spacing 400 μm) showing the pattern of nerves and the cohesion of axons innervating EMUc in a normal limb. In the centre is shown the nerve pattern (filled profiles), the position of the cartilage elements (hatched profile) and the injection site (solid area in bottom section). At the sides are shown at higher magnification the nerves with filled axons (spacing 200 μm) through the plexus (left) and near the injection site (right). The ventral nerves have no filled axons and complicate the figure, they have therefore been discontinued past the arrow on the left side. The host axes are dorsal upwards and anterior to the right. Bar, 50 μm for whole limb and 100 μm for nerve sections. The filled axons (from the caudolateral pool in the motor horn see Figs 2, 3A) maintain their posterior position within the plexus and radial nerve; they move closer together throughout their course down the limb (see Fig. 6A,C).

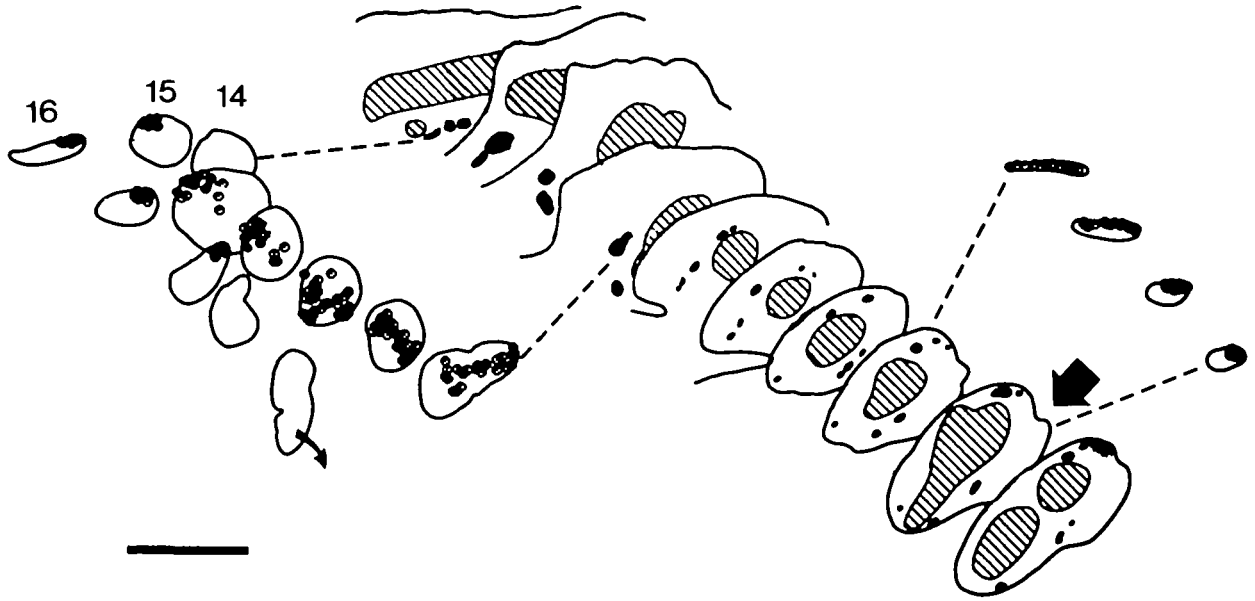


Fig. 5. Computer-generated reconstruction from serial sections of the duplicated limb of animal shown in Fig. 4: arrangement, orientation and conventions as Fig. 4. EMUa was injected, filling axons from the caudolateral pool (see Fig. 3A). On the left side, we see that proximally these occupy dorsal posterior sectors of the 15th and 16th spinal nerves, but at the plexus they have already begun to move so that by the last section most are in the anterior sector of the radial nerve leaving an unfilled region which will go to triceps muscle. At this point (see centre panel), about a third of the way down the humerus, the radial splits up into several branches, the most posterior innervating the triceps muscle. The labelled axons are situated in the middle branch over the humerus, which further distally becomes flattened (enlargement on the right and Fig. 6B). The filled axons then collect together to innervate EMUa (see Fig. 6D). The large arrow marks the level at which the skeletal and muscle pattern is clearly duplicated.

the radial so that the position of the axons within the nerve presages the position of their ultimate targets. After the branches to triceps have left, the filled axons occupy the extreme posterior sector of the radial as it travels over the humerus. In the forearm, the branch to the EMUc muscle arises posteriorly. The filled axons therefore maintained their posterior position within the radial nerve with respect to their neighbours (Fig. 6A,C).

On the operated side (Fig. 5), the axons filled from an EMUa injection also occupy the dorsal segments of 15th and 16th nerves proximal to the plexus (from the lateral motoneurones in caudal lateral motor column, Figs 2, 3A,B). However, the filled axons do not maintain this position further distally. Where the brachial plexus divides into dorsal and ventral branches the filled fibres are already spread over the whole radial nerve and are not localized as on the unoperated side (compare Fig. 4), although at this level the limb structure (skeleton, muscles, tendons, dermal structures) looks normal until the elbow. About halfway down the humerus, the triceps branches leave as usual, at this point the radial nerve divides abnormally into three branches. It is difficult to name these anterior branches because they do not go to identifiable targets; in the duplicated limb there is no alar web and no anterior extensor (EMR). There are no labelled axons in these anterior branches. The posterior branch containing filled axons continues distally in the position characteristic for the radial nerve. This nerve becomes very compressed at this point and is often surrounded by part of the matrix surrounding the humerus. Here it is too deep within the tissue for the chromogen to penetrate so that filled axons are not visible. Further distally where the chromogen has penetrated, the filled axons are found throughout the nerve where they are again visible (Fig. 6B), further down they coalesce towards the injection site to leave in a tight clump anteriorly as is shown in Fig. 6D and in the enlarged reconstruction to the right (Fig. 5). The large arrow shows the first section in which the underlying pattern of the limb is clearly duplicated.

The pattern of nerves in the other limbs containing labelled axons was similar. Immediately distal to the plexus, filled axons occupied positions characteristic for EMUc in a normal limb. Filled axons move to more anterior positions within the nerve at more distal levels. Further distally the radial nerve splits into several branches with further transposition of axons to reach the anterior duplicated muscle. In all cases, the localization of axons and the nerve pattern were abnormal far proximal to duplication of skeleton, muscles or dermal structures (see below). In

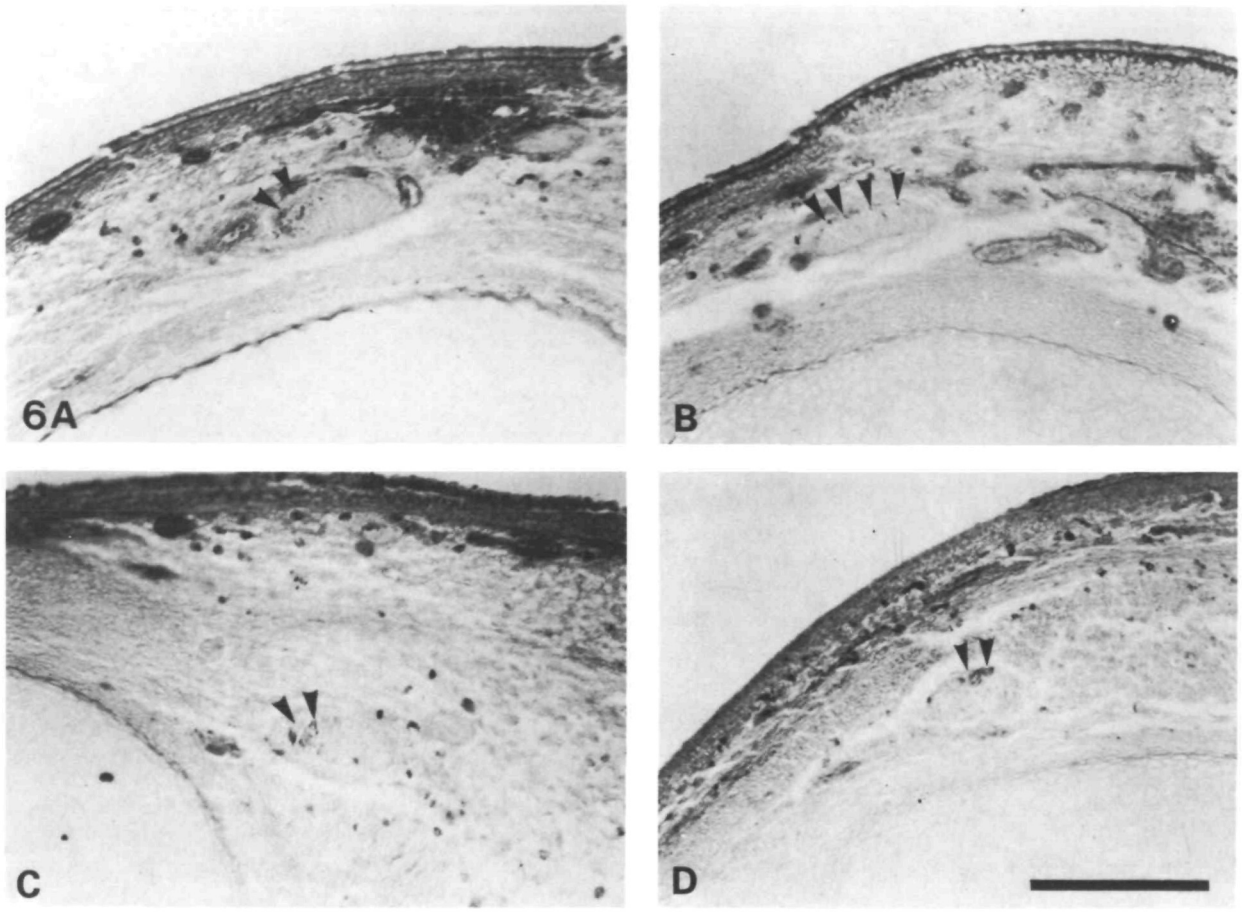


Fig. 6. Micrographs of transverse sections through unoperated (A,C) and duplicated limb (B,D) reconstructed in Figs 4 and 5 half-way down humerus and just before muscle nerve leaves to injected muscle. Host axes dorsal upwards anterior to the right. Bar, 100 μm. (A) Filled axons from EMUc injection (arrowheads) lie in the posterior sector of the radial nerve. (B) In the duplicated limb, the axons from EMUa injection are spread right across the radial nerve. (C) The filled axons from EMUc injection collect together posteriorly just proximal to the muscle nerve exit point. (D) Further distally, close to the branch to EMUa, the filled axons have collected together anteriorly and none are seen in the posterior sector of the nerve.

normal limbs, the position of axons within a nerve presages the position of their targets in the limb. Thus, when the anterior forearm extensor muscle EMRc is injected with HRP, the filled fibres occupy a more anterior position in the radial nerve. In Fig. 7, they can be seen running in a band across the radial nerve, the axons anterior to them are sensory axons which leave the radial to innervate the alar web *via* the patagialis nerve. Distal to this point, the filled axons are far anterior in the radial from which position the nerve to the EMR muscle arises. The axons do not change their relative positions within the nerve, their trajectories are clearly related to the topological position of EMRc and unlike those seen in duplicated wings, EMRc injections never filled axons in spinal nerve 16.

Three limbs were stained with the Bodian silver method following HRP labelling. None of the limbs had perfect symmetrical mirror-image duplication of the nerve pattern. In all three, the dorsal nerve spread out into a skein proximally where surrounding tissue was still unduplicated; beyond the elbow where muscles and skeleton were clearly reduplicated the nerve pattern was mirror-imaged.

The nerve pattern in the three animals where the pool of the EMUa muscle was found in cranial rather than caudal cord was clearly different to the patterns described above. In all three cases the ZPA graft operation was performed at an early stage (stage 17/18) and, in all three, abnormalities of both skeletal, muscle and nerve pattern occurred at the head of the humerus rather than towards the elbow. Fig. 8 illustrates how the three segmental nerves enter the limb and immediately form a ring of nerves surrounding the head of the humerus. There is no normal plexus and the segmental nerves do not obviously join together. The filled axons from the EMUa injection are found in the anterior nerves in the limb which are

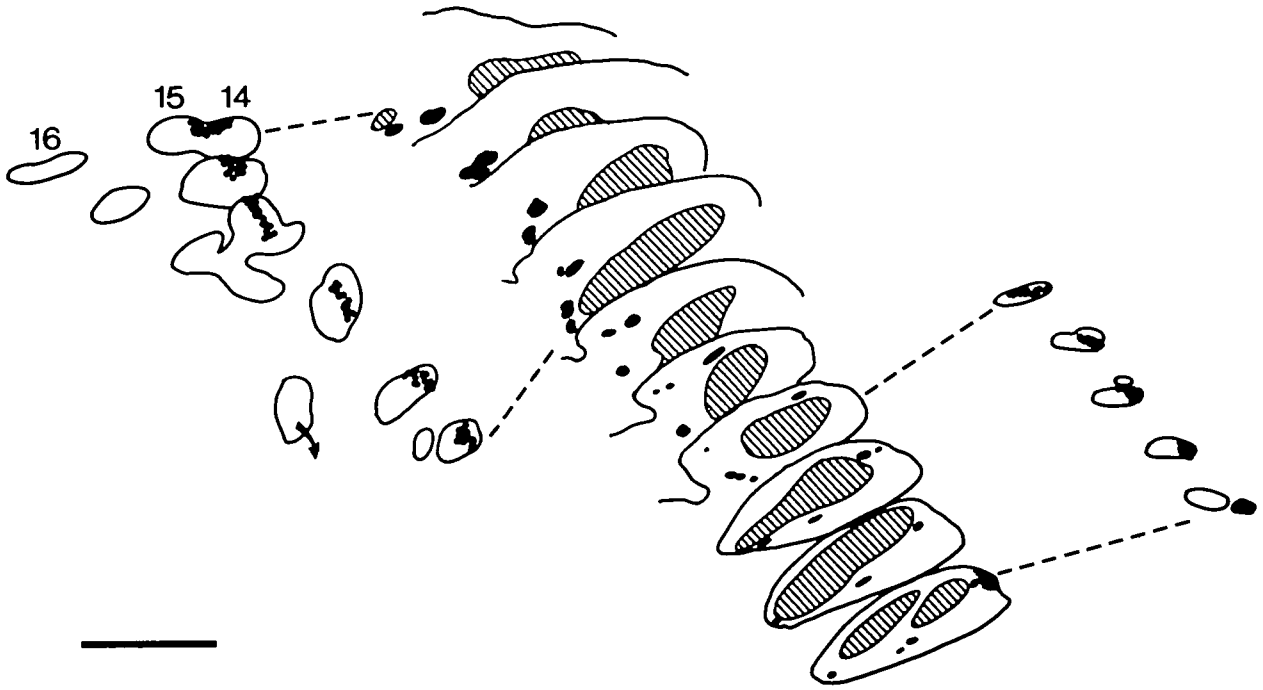


Fig. 7. Reconstruction from serial sections of limb (centre) and nerves (right and left) of the unoperated limb of animal illustrated in Fig. 3B in which the anterior EMR muscle was injected with HRP. Conventions as for Figs 4 and 5, host axes dorsal upwards and anterior to the right. Bar, 500 μm for limb and 100 μm for nerves.

Axons from the rostrolateral motor pool lie dorsally in spinal nerves 14 and 15, they maintain their position in the anterior sector of the radial nerve. Halfway along the humerus (to the left) the unfilled sensory nerve to the alar web branches, the filled axons then collect together to innervate the anterior EMR muscle.

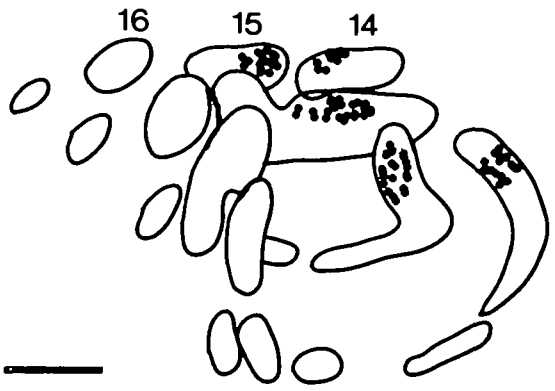


Fig. 8. Reconstruction from serial sections showing pattern of nerves and filled axons in a very proximally duplicated limb in which EMUa was injected with HRP. Section spacing 50 μm, host axes dorsal upwards anterior to the right. Bar, 100 μm.

Filled axons from the motor pool in rostrolateral motor horn (as in Fig. 3B unoperated side), occupy the dorsal anterior sector of spinal nerve 15 and the dorsal posterior sector of nerve 14. The plexus is very abnormal, the 16th spinal nerve does not join 14 and 15 before all nerves divide dorsoventrally to surround the enlarged head of the humerus. The axons from caudal motoneurones cannot reach the EMUa, which is innervated by cells in the rostral cord.

continuous with the 14th segmental root. The division of the dorsal nerve into many small branches resulted in filled fibres being found entirely filling several small branches so that there was no opportunity of seeing if their position within the nerves changed more distally.

Pattern of limb tissues and level of reduplication

Serial sections of all wings in which filled axons could be traced as far as the plexus were examined to determine the level of reduplication of the underlying structure of the wing. The skeleton, muscles, tendons and feather germs were used as markers for the level of duplication and mapped onto the *camera-lucida* drawings used for three-dimensional reconstruction. Sections were scored as having normal symmetry when all four markers had anatomical relationships similar to an equivalent section from the control side. In all cases, sections distal to the elbow were clearly in mirror-image symmetry about the midline. Proximal to this was a narrow zone where the morphology of the limb was disturbed showing neither mirror-image symmetry nor a normal pattern of tissues. Further proximally the pattern of tissues was normal. The gross anatomy of the nerves on the whole followed the pattern of the underlying tissue but the region of

disturbed anatomy tended to be slightly more proximal than for the other four markers. These data were used to indicate the most proximal section at which the anatomy of the experimental limb was abnormal (Fig. 5).

Distribution of filled dorsal root ganglion cells from EMUa injection

Filled dorsal root ganglion cells were seen in 17 animals after injection of the EMUa muscle. In 11 of these, the ganglia labelled on the two sides were symmetrical and corresponded to the position of the labelled motor pool. In six cases the labelling was not symmetrical, on the unoperated side 15th and 16th DRGs were labelled as expected while on the operated side the 14th and 15th DRGs were labelled, in the other three only the 14th DRG was labelled on the operated side.

Positions of motoneurones innervating posterior EMUp muscle

In two animals with duplicated wings, the posterior EMUp muscle was injected with HRP. Although there were fewer labelled motoneurones on the operated side in both cases, they occupied the caudolateral position in the motor horn characteristic for EMU muscle injection in unoperated wings. Both the posterior (EMUp) and anterior (EMUa) muscles were injected in three operated animals. Despite having injected two muscles in the duplicated wing there were fewer cells labelled than on the control side, but again their position was characteristic of single EMUc injection in normal wings. We could not follow trajectories in these animals well enough to discover the point at which axons diverged to innervate posterior and anterior target muscles.

Trajectories of orthogradely filled motor axons

Since the trajectories of axons filled from muscle injection were hard to follow, we tracked the position of motor and sensory axons labelled from localized injection into the spinal cord and dorsal root ganglia. For such studies it was important to compare the position of filled axons at equivalent positions along the nerves on unoperated and operated sides, therefore the injections needed to be discrete and equivalent on the two sides. Injection of the cord at the level of the 14th spinal nerve in two operated animals filled fibres that maintained their anterior position within the nerves of the duplicated limb. Axons filled from cord injections rarely travelled further than the elbow so that we were unable to show that they innervated any particular forearm muscle. Injection of 16th dorsal root ganglion in five cases labelled axons that retained their position in the radial and medio-ulna nerves, innervating posterior targets in the limb. We injected the 16th spinal cord segment in 19 animals with good duplicated limbs; in 7 reasonably matched injections axons were filled to the elbow and there were clear signs of a spread (rather than a shift) of filled fibres on the operated side (Figs 9, 10). The filled axons occupying the most-anterior ventral nerve on the operated side in a couple of cases had moved across from the medio-ulnar nerve more proximally where the two nerves ran close together and then diverged again. Such anastomoses are not seen in normal limbs, but were quite common in the duplicated limbs. Filled axons could not be followed in the radial nerve beyond the elbow so that their distribution to duplicated targets is not known.

Discussion

The purpose of these experiments was to observe the behaviour of motor axons growing into limbs whose structure had been duplicated across the anteroposterior axis by a ZPA graft. Two groups of cells in two places that normally become two different muscles instead became the same muscles. The position of motoneurones innervating the anterior duplicate of the posterior wrist extensor muscle clearly showed that some of the motor axons from the posterior extensor motor pool had found their way to the new 'appropriately' named target despite its anterior position and despite the continuing presence of the original 'appropriately' named target in the normal position. What surprised and excited us was how they got there.

Recent evidence has favoured the idea that axons respond to guidance cues closely associated with their immediate surroundings (Lewis, 1978; Summerbell & Stirling, 1981; Taghert & Lichtman, 1986; Tosney & Landmesser, 1985*a*,*b*). This is best illustrated by the analogy to a public highway (Lewis *et al.* 1983; Summerbell & Stirling, 1981). The concept of a highway implies the existence of a paved surface and it remains fasionable to look for extracellular materials that may provide a surface over which the axons prefer to grow.

We have recently argued (Stirling & Summerbell, 1987) that the concept of a highway is misleading. First, the mature nerve pattern does not necessarily reflect the behaviour of growing axons since the embryonic appearance is considerably modified by subsequent limb development such as glial bundling, differential growth and limb torsion. Second, the growing nerve front occupies most of the available connective tissue being excluded only from impenetrable tissues such as cartilage. Third, while the pattern and size of the limb skeleton is accurately controlled (Summerbell & Wolpert, 1973), the pattern of nerves is constant only in the sense that axons

run predominantly in a proximodistal direction between other major anatomical features and there is a fairly constant number of branches.

Our new results show how both the morphological pattern of nerves and the relative positions of particular axons within the nerves adapts to a distant rearrangement of targets even though the surrounding structure through which they are growing (muscles, skeleton and connective tissues) appears to be normal. This makes it less likely that axons are responding solely to localized overt differentiation products such as trails of laminin and/or fibronectin

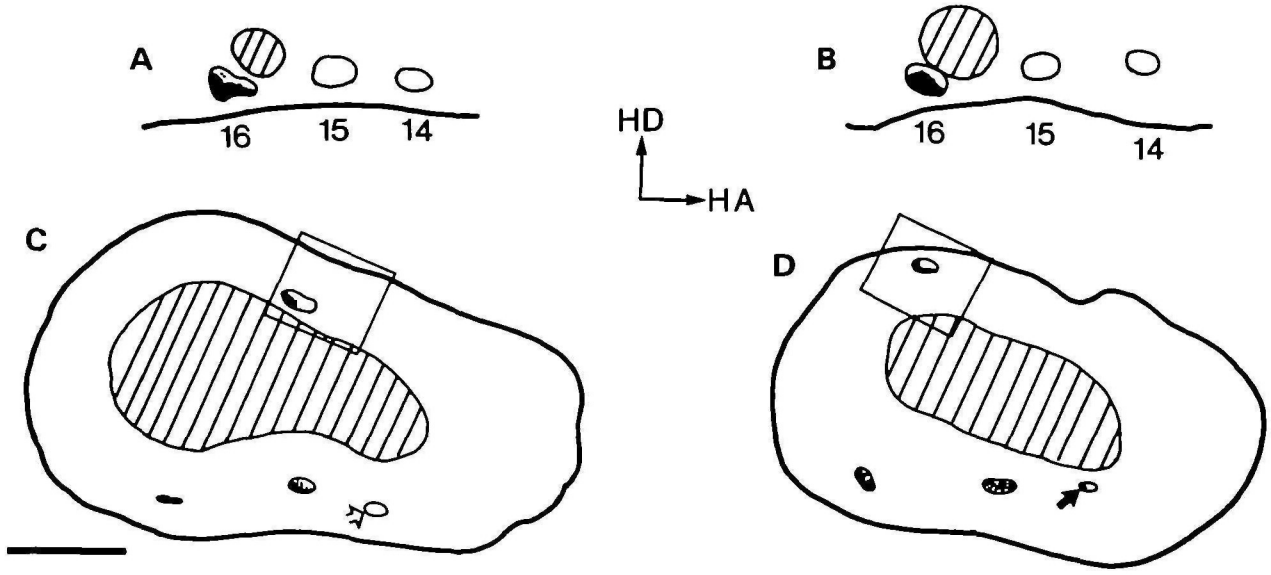


Fig. 9. Camera-lucida drawings of sections at the level of the spinal nerves (A,B) and halfway down the humerus (C,D) of unoperated (A,C) and duplicated (B,D) limbs in an animal in which HRP was injected into the ventral nerve cord to fill 16th spinal nerve. Boxes show position of micrographs in Fig. 10. Host axes dorsal upwards anterior to the right. Bar, 100 μm. (A,B) Injection fill ventral sector of 16th spinal nerve on both sides. (C) On the unoperated side, the filled axons lie in the posterior sector of the radial nerve (Fig. 10) and are also present in the ulnar and median nerves, but not the biceps nerve (open arrow). (D) In the duplicated limb, the axons are not confined to the posterior sector of the radial nerve. All three ventral nerves contain axons, those in the most anterior nerve (anterior duplicate of ulna nerve, solid arrow) moved across from the median at an anastomosis which is common in duplicated limbs but rarely seen in normal limbs.

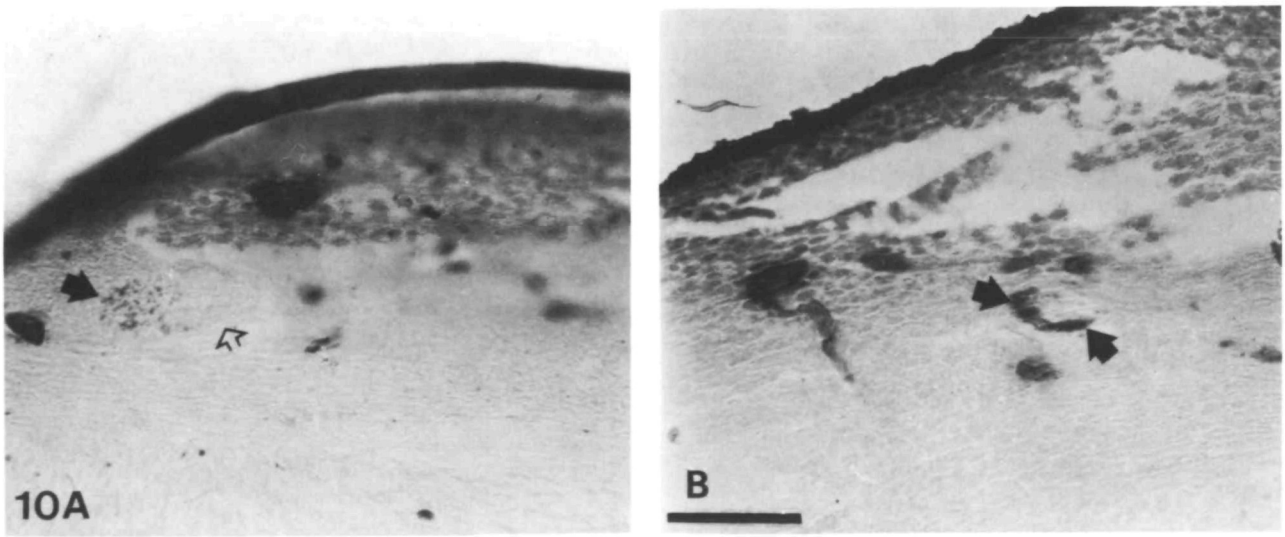


Fig. 10. Photomicrographs of the radial nerve at positions outlined in Fig. 9C,D. Bar, 100 μm. (A) Unoperated limb, filled axons in posterior sector of radial nerve (solid arrow) none in the anterior sector (open arrow). (B) Duplicated limb, the filled axons from a matched injection (Fig. 9A,B) are not confined to the posterior sector of the radial nerve (arrows).

and more likely that they are using long-range signals or obeying the basic rules that specify pattern formation within the tissues.

The point of decision

In normal limbs, the axons innervating a particular muscle occupy a characteristic position in the plexus and come from a localized pool of cells in the cord which is topologically related to the position of the target muscle in the limb (Horder, 1978; Stirling & Summerbell, 1985). Axons maintain their position relative to neighbours as they travel down the limb to the muscle nerve exit (Hardman & Brown, 1985; Laskowski & Sanes, 1987). Here there may be some sorting out of axons (Tosney & Landmesser, 1985*b*). It has been suggested that this could be in response to a short-range signal from the target muscle (Lewis *et al.* 1981), or local cues or 'guidepost' cells at the branch point (Tosney & Landmesser, 1985*b*; Bastiani *et al.* 1985). In our duplicated limbs, some of the motor axons from the caudolateral pool do not maintain their normal position in the radial nerve but instead start to change their position in the nerve soon after it leaves the plexus. The surrounding tissues of the limb still appear entirely normal and in many cases signs of duplication are seen only at the level of the elbow. By the time the axons reach the branch down which they will travel to reach the duplicated EMU, they are already bundled in an appropriate position and no major sorting out is required. Clearly the change in the trajectory of the axons is not dependent on local interactions. The presence of the ZPA has in some way changed the environment of the axons at a position in the limb where it is unable to modify the overt differentiation of the limb.

Positional value and nonequivalence

The theory of positional information (Wolpert, 1969) supposes that cells can have their position specified within an embryonic field using long-range signals from special organizing regions. This positional value is recorded within the cell and later used to modify gene expression so that the cell differentiates in an appropriate way for its position within the anatomical pattern of the field or embryo. This theory has proved powerful and robust and we have learned a great deal about both positional signals and the control of gene expression. Indeed, most readers will tend to think of these as fact rather than theory. However, there is still no direct evidence that between these two phases there exists a time at which the information is present but not yet enacted. The evidence has been reviewed frequently and the cell state involved described variously as for example: specification (Wolpert, 1969), positional memory (Summerbell & Tickle, 1977; Smith, 1979), nonequivalence (Lewis & Wolpert, 1976; Summerbell, 1976) or hidden anatomy ('secret name', Slack, 1982). All describe the same basic feature: that two cells that appear identical (they have the same genome and express the same structural genes) are nevertheless in some way different. The best evidence for this cell state so far is the demonstration of spatially restricted patterns of staining with monoclonal antibodies in uniform embryonic fields. The only examples that we know of are *Drosophila* imaginal discs (Wilcox *et al.* 1981) and chick wing buds (Ohsugi & Ide, 1986). Such invisible labels could play a role in neuronal specificity (Sperry, 1963).

The axons innervating EMUa behave as if the cells of this muscle are EMU cells and not simply cells with a muscle phenotype. If we assume (Beresford, 1983; Christ *et al.* 1977; Chevalier *et al.* 1977) that the reduplicate muscle is made up of the same cells that would have formed the extensor metacarpi radialis (EMR) in a normal limb, then the operation has in effect changed the name of these cells even though they have not overtly changed their phenotype. This, so far as we know, is the first demonstration that a positional signal (in this case from the ZPA) can change a hidden cell state (perhaps the positional value) as well as the overt pattern of differentiation. We know of many cases, including ZPA grafts, in which one can modify the anatomy of an embryo by changing positional signals. It is not clear to what extent this involves spatial rearrangement of a limited repertoire of cell phenotypes and to what extent it involves the hypothetical hidden cell state. In this case, the anterior muscle cells overtly differentiate into the same range of cellular phenotypes that would have been present in an unmanipulated embryo but there is clearly another more subtle change. So far we cannot characterize or recognize this difference, but the axons can. Like Roland Young's lovelorn fleas '...she can tell and so can he' (in *Not for Children*, Doubleday).

Given that this experiment demonstrates a change in positional value then it seems plausible that the axons are also using the same positional values to navigate through the limb bud. The axons cannot be responding to a new pattern of differentiation in the proximal tissues. At the level at which we see the trajectories changing, the overt pattern of muscles, cartilage and tendons is normal and the pattern has been determined for some time (Hornbruch, 1980; Summerbell, 1974, 1977; Summerbell & Lewis, 1975). This means that the limb cells are still able to change their positional identity (value) at a time when the pattern of differentiation (or gene expression) is already determined.

Direct control by positional signals

The form of the limb (wing or leg) is determined by the origin of the limb mesenchyme (wing or leg) and is independent of the origin of the limb ZPA (Summerbell & Tickle, 1977). The anatomical pattern of nerves within the limb is also specific to the limb mesenchyme origin. Wing axons growing into a leg bud form a leg pattern of nerves (Summerbell & Stirling, 1983). The pattern of nerves cannot therefore be determined only by a direct interaction between axons and the signal from the ZPA.

However, the distribution of individual motor axons within the nerve bundles may not be limb specific. There is an obvious topological similarity between the distribution of motor axons to fore- and hindlimbs. Anterior motor pools innervate anterior targets and medial motor pools innervate ventral targets etc. This observation leaves the possibility that the relationship between the motor pool map in the cord and the position of axons in the nerves is controlled directly by the positional information signal rather than indirectly by positional values in the periphery. Axons from caudal pools adopt a more posterior position in the plexus and limb nerves i.e. they travel at a higher end of the concentration gradient of the ZPA signal than their more rostral neighbours. When the limb bud is reversed about the AP axis these axons will alter their position in the nerve in relation to the new position of the ZPA signal. This change in their position is not confined to branch points or the plexus but occurs throughout their course through either the fore- or hindlimb. Such a model in which axons are sensitive to an orientation signal is similar to that proposed to explain the development of the retinotectal projection (Hope *et al.* 1976).

Long-range signals from the target

Long-range attraction by target muscles (chemotaxis) is unlikely to play a significant role. One does occasionally observe unique branches from the main nerve trunks that carry axons to appropriate targets. These are usually seen when the path of the nerve carries axons near to 'familiar' territory (Stirling & Summerbell, 1983). Specific examples are described in Lance-Jones & Landmesser, 1980; Summerbell & Stirling, 1981. Similarly, there is evidence that in normal limbs motor axons form branches from the main nerve trunks when they pass nearby developing muscles (Lewis *et al.* 1981). The range of these interactions seems very short (200 μm or less). In these experiments, it seems likely that the decisions on trajectories are being taken both before the target muscle mass has started to form and distant from its location. Axons are entering the region where trajectory correction takes place from stage 22 (Hollyday, 1983; Roncali, 1970). The target muscle will not individuate until at least stage 29, about 60 h later (Shellswell & Wolpert, 1977). Nor are the axons following the myoblasts as they move together to form the muscle, the axons show no specific preference for myoblasts from a particular origin (Keynes *et al.* 1987). It is difficult to estimate from our data the distance between correction of trajectory and the target because of the differential growth rates of limb and nerves but the distance between trajectory correction and the position at which the branch point to EMUa will form is unlikely to ever be less than 500 μm.

Sensory projection

The injections into the cord or dorsal root ganglia (DRG) distinguish the orthograde trajectories of motor and sensory fibres, respectively. While there was no obvious shift in the position of filled sensory axons from the DRG of spinal nerve 16, there were definite signs of modified trajectories of motor axons from injections into the 16th motor horn. Following muscle injections, filled DRG cells (presumably from proprioceptor afferents) were found in some animals. In most of these, the distributions of labelled cells were similar on operated and control sides, but in a few there was a definite mismatch with labelled cells in the 14th DRG on the operated side. It is possible that in these cases some cutaneous afferents had taken up the HRP. This raises the possibility that cutaneous axons may not be under the same growth constraints as motor or proprioceptive axons. Recent experiments by Honig *et al.* (1986) and Scott (1986) comparing the pattern of motor and proprioceptive axons in experimental animals suggest that at least the pattern of peripheral innervation of the latter may be controlled by motor axons. The difference in the guidance of proprioceptive and cutaneous fibres reflects the difference in how they make their central connections. Proprioceptive afferents make accurate synaptic connection with motoneurones from the outset (Frank & Westerfield, 1983) while cutaneous projections to dorsal horn neurones are less precise early in development (Fitzgerald, 1985).

Conclusion

We still find it astonishing that axons modify their trajectories at a position in the limb where there is no overt change in the pattern of differentiation of the limb cells. If the observation stands up to further investigation it will significantly modify the way in which we think about the control of limb innervation. At the same time, the data provide the best information yet that a cell is able to change some internal characteristic, the positional value, that can be recognized by another cell (or its axon) without leading to a

change in the cell's phenotype. This provides significant support for the theory of positional information.

We wish to thank John Brassey and Eileen Clark for expert technical advice and assistance, Roger Keynes for advice on the manuscript and Fiona Harvey who first persuaded us that the experiment was interesting.

References

Bastiani, M. J., Doe, C. Q., Helfand, S. L. & Goodman, C. S. (1985). Neuronal specificity and growth cone guidance in grasshopper and Drosophila embryos. *TINS* **8**, 257–267.

Beresford, B. (1983). Brachial muscles in the chick embryo: the fate of individual somites. *J. Embryol. exp. Morph.* **77**, 99–116.

Chevallier, A., Kieny, M. & Mauger, A. (1977). Limb-somite relationships: origin of the limb musculature. *J. Embryol. exp. Morph.* **41**, 245–258.

Christ, B., Jacob, H. J. & Jacob, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. *Anat. Embryol.* **150**, 171–186.

Fitzgerald, M. (1985). The post-natal development of cutaneous afferent fibre input and receptive field organisation in the rat dorsal horn. *J. Physiol.* **364**, 1–18.

Frank, E. & Westerfield, M. (1983). Development of sensory-motor synapses in the spinal cord of the frog. *J. Physiol.* **343**, 593–610.

Hamburger, V. M. & Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

Hardman, V. J. & Brown, M. C. (1985). Axon pathways within peripheral nerves in the rat hind limb. *N. Sci. Letts. suppl.* **22**, S333.

Holliday, M. (1983). Development of motor innervation of chick limbs. *Prog. Clin. Biol. Res.* **110A**, 183–193.

Honig, M., Lance-Jones, C. & Landmesser, L. (1986). The development of sensory projection patterns in embryonic chick hindlimb under experimental conditions. *Devl Biol.* **118**, 532–548.

Honig, L. S. & Summerbell, D. (1985). Maps of strength of positional signalling activity in the developing chick wing bud. *J. Embryol. exp. Morph.* **87**, 163–174.

Hope, R. A., Hammond, B. J. & Gaze, R. M. (1976). The arrow model: retinotectal specificity and map formation in the goldfish visual system. *Proc. R. Soc. Lond.* B. **194**, 447–466.

Horder, T. J. (1978). Functional adaptability and morphogenetic opportunism the only rules for limb development? *Zoon* **6**, 181–192.

Hornbruch, A. (1980). Abnormalities along the proximodistal axis of the chick wing bud: the effect of surgical intervention. In *Teratology of the Limbs* (ed. H.-J. Merker, H. Nau & D. Neubert), pp. 191–197. Berlin: De Gruyter.

Keynes, R., Stirling, R. V., Stern, C. D. & Summerbell, D. (1987). The specificity of motor innervation of chick wings does not depend upon the segmental origin of muscles. *Development* **99**, 565–575.

Lance-Jones, C. & Landmesser, L. (1980). Motoneurone projection patterns in the chick hind limb following early partial reversals of the spinal cord. *J. Physiol.* **302**, 581–602.

Landmesser, L. T. (1978). The distribution of motoneurones supplying chick hind limb muscles. *J. Physiol.* **284**, 371–389.

Laskowski, M. B. & Sanes, J. R. (1987). Topographic mapping of motor pools into skeletal muscles. *J. Neurosci.* **7**, 252–260.

Lewis, J. (1978). Pathways of axons in the developing chick wing: evidence against chemo-specific guidance. *Zoon* **6**, 175–179.

Lewis, J. H., Al-Ghaith, L., Swanson, G. & Khan, A. (1983). The control of axon growth in the developing chick wing. *Prog. Clin. Biol. Res.* **110A**, 195–205.

Lewis, J. H., Chevallier, A., Kieny, M. & Wolpert, L. (1981). Muscle nerve branches do not develop in chick wings devoid of muscle. *J. Embryol. exp. Morph.* **68**,149–160.

Lewis, J. H. & Wolpert, L. (1976). The principle of non-equivalence in development. *J. theor. Biol.* **62**, 479–490.

Neset, M., Muniak, C. & Edwards, C. (1985). Studies of innervation patterns in grafted chick wings. *J. comp. Neurol.* **232**, 70–82.

Ohsugi, K. & Ide, H. (1986). Position specific binding of a monoclonal antibody in chick. *Devl Biol.* **117**, 676–679.

Roncali, L. (1970). The brachial plexus and the wing nerve pattern during early developmental phases in chicken embryos. *Monitore Zool. Ital.* **4**, 81–98.

Saunders, J. W. Jr. & Gasseling, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. In *Epithelial–mesenchymal interactions* (ed. F. Fleischmajer & R. E. Billingham), pp. 78–97. Baltimore: Williams & Wilkins.

Scott, S. A. (1986). Skin sensory innervation patterns in embryonic chick hind limb following dorsal root ganglion reversal. *J. Neurobiol.* **17**, 649–668.

Shellswell, G. B. & Wolpert, L. (1977). The pattern of muscle and tendon developments in the chick wing. In *Vertebrate Limb and Somite Morphogenesis* (ed. D. A. Ede, J. R. Hinchliffe & M. Balls), pp. 71–86. Cambridge University Press.

Shepherd, A. M., Perkins, W. J., Green, R. J. & Clark, S. A. (1984). A study of oesophageal structure in a nematode, *Asphelenchoides blastophthorus (Aphelenchida)*, using computer graphics reconstruction from serial thin sections. *J. Zool.* **204**, 271–288.

Slack, J. M. W. (1982). Regeneration and the second anatomy of animals. In *Developmental Order* (ed. S. Subtelny & P. B. Green), pp. 423–436. London: Academic Press.

Smith, J. C. (1979). Evidence for a positional memory in the development of the chick wing bud. *J. Embryol. exp. Morph.* **52**, 105–113.

SPERRY, R. W. (1963). Chemoaffinity in the orderly growth of nerve fibre patterns and connections. *Proc. natn. Acad. Sci. U.S.A.* **50**, 703–710.

STIRLING, R. V. & SUMMERBELL, D. (1983). Familiarity breeds contempt: the behaviour of axons in foreign and familiar environments. *Prog. Clin. Med. Res.* **110A**, 217–226.

STIRLING, R. V. & SUMMERBELL, D. (1985). The behaviour of growing axons invading developing chick wing buds with dorsoventral or anteroposterior axis reversal. *J. Embryol. exp. Morph.* **85**, 251–269.

STIRLING, R. V. & SUMMERBELL, D. (1987). Motor axon guidance in the developing chick limb. In *The Making of the Nervous System* (ed. J. G. Parnavelas, C. D. Stern & R. V. Stirling), pp. 000–000. Oxford University Press.

SUMMERBELL, D. (1976). A descriptive study of the rate of elongation and differentiation of the skeleton of the developing chick wing. *J. Embryol. exp. Morph.* **35**, 241–260.

SUMMERBELL, D. (1974). Interaction between the proximo-distal and antero-posterior coordinates of positional value during the specification of positional information in the early development of the chick wing bud. *J. Embryol. exp. Morph.* **32**, 227–237.

SUMMERBELL, D. (1977). Regulation of deficiencies along the proximal distal axis of the chick wing bud: a quantitative analysis. *J. Embryol. exp. Morph.* **35**, 241–260.

SUMMERBELL, D. & LEWIS, J. H. (1975). Time, place and positional value in the chick wing bud. *J. Embryol. exp. Morph.* **33**, 621–643.

SUMMERBELL, D. & STIRLING, R. V. (1981). The innervation of dorsoventrally reversed chick wings: evidence that motor axons do not actively seek out their appropriate targets. *J. Embryol. exp. Morph.* **61**, 233–247.

SUMMERBELL, D. & HORNBRUCH, A. (1981). The chick embryo: a standard against which to judge *in vitro* systems. In In Vitro *Studies of Prenatal Differentiation and Toxicity* (ed. D. Neubert & H. Merker), pp. 529–538. Berlin: de Gruyter.

SUMMERBELL, D. & TICKLE, C. (1977). Pattern formation along the antero-posterior axis of the chick limb bud. In *Vertebrate Limb and Somite Morphogenesis* (ed. D. A. Ede, J. R. Hinchliffe & M. Balls), pp. 41–53. Cambridge University Press, Cambridge.

SUMMERBELL, D. & WOLPERT, L. (1973). Precision of development in chick limb morphogenesis of the chick limb. *Nature New Biology, Lond.* **238**, 24–25.

TAGHERT, P. H. & LICHTMAN, J. W. (1986). Axon outgrowth in vertebrates. *Nature, Lond.* **320**, 210–211.

TICKLE, C., SUMMERBELL, D. & WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature, Lond.* **254**, 199–202.

TOSNEY, K. W. & LANDMESSER, L. T. (1985*a*). Specificity of early motoneuron growth cone outgrowth in the chick embryo. *J. Neurosci.* **5**, 2336–2344.

TOSNEY, K. W. & LANDMESSER, L. T. (1985*b*). Growth cone morphology and trajectory in the lumbosacral region of the chick embryo. *J. Neurosci.* **5**, 2345–2358.

WILCOX, M., BROWER, D. L. & SMITH, R. J. (1981). A position-specific cell surface antigen in the *Drosophila* wing imaginal disc. *Cell* **25**, 159–164.

WOLPERT, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. theor. Biol.* **25**, 1–47.

(*Accepted 2 February 1988*)