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The innervation of dorsoventrally reversed chick wings: evidence that motor axons do not actively seek out their appropriate targets

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SUMMARY

In normal chick embryos the extensor (dorsal) muscles are innervated by motoneurones lying laterally in the motor horn, while flexor muscles are supplied by more medially placed motoneurones. After reversal of the dorsoventral axis of the forelimb prior to innervation in most cases the opposite pattern is found, the extensors innervated by medial and flexors by lateral motor neurones. In a minority of cases the normal innervation pattern is obtained.

Three hypotheses are discussed, two involving specific target affinity between motor axon and target and one involving passive deployment of axons to targets. We conclude that our results favour the latter hypothesis but that we cannot exclude a short-range specific signal.

INTRODUCTION

When spinal nerves innervate the developing chick limb they form repeatable and ordered patterns. Recent interest has concentrated on three of these patterns: the anatomical arrangement of nerves within the limb (Narayanan, 1964; Stirling & Summerbell, 1977; Lewis, 1978); the distribution of axons from different segmental roots to different limb territories (Landmesser & Morris, 1975; Stirling & Summerbell, 1979; Pettigrew, Lindeman & Bennett, 1979; Bennett, Lindeman & Pettigrew, 1979); and the arrangement of motor pools within the cord supplying specific muscles (Hollyday & Hamburger, 1977; Landmesser, 1978*a*, *b*). In this paper we provide evidence that much of this pattern may be explained by passive deployment of axons without specific target affinity.

The importance of passive guidance in the development of innervation pattern has been widely discussed for a long time (e.g. His, 1887; Harrison, 1910; Weiss, 1941), and more recently (Lewis, 1978: Horder, 1978; Stirling & Summerbell, 1979). This contrasts with theories that involve the establishment of pre-determined matching between the neuron and its target (e.g. Cajal, 1910; Sperry, 1963) of which we will consider two current versions. The first suggests

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that the axons are initially specified with respect to their peripheral destination and that they seek out and connect with their appropriate targets (Landmesser & Morris, 1975). The other version supposes that initial innervation is totally random with axons reaching haphazardly any possible target. Only those innervating their matched destination survive – the remainder die, as seen in the period of massive cell death (Lamb, 1977; Pettigrew, *et al*, 1979). Other hypotheses and the early history have been well reviewed by Landmesser & Morris (1975) and Horder (1978).

Our passive deployment model (Stirling & Summerbell, 1979) arose from our observations on the nerve patterns in rotated chick wings. We rotated chick wing buds 180° about the proximodistal axis before the axons reached them and observed the anatomy and the segmental distribution of nerves to the wing. We found that the anatomy of the graft and its nerves was normal (axes rotated relative to host). There was no indication of selective innervation: so that the anterior segmental nerves innervated targets normally supplied by the posterior segmental nerves and vice versa. Thus the segmental distribution was reversed.

However, since we did not know the way the axons behaved within the cord, we could not rule out some kind of axon selectivity within the cord itself. In this paper we describe experiments in which motoneurone pools supplying particular muscles were mapped using the Horseradish peroxidase (HRP) retrograde labelling technique (Mesulam, 1976; Lamb, 1977; Landmesser, 1978*a*, *b*; Bennett *et al.* 1979).

METHODS

Fertilized eggs from a local flock (Needle Farm) were incubated at 38 °C and windowed on day 3. The windows were sealed with Sellotape and the eggs returned to the incubator. We selected pairs of embryos within half a stage (Hamburger & Hamilton, 1951) of each other between stages 18 to 20 inclusive, long before the axons reach the bud (Roncali, 1970). The left wing bud of one embryo was exchanged for the right wing bud of the other embryo so that both grafts had the dorso-ventral axis reversed and the anterior–posterior axis unchanged. The intended level of reversal was either at the elbow or the shoulder as judged from data in Stark & Searles (1973) and Summerbell (1977). In the other case we performed a very similar operation but this time transferred the right wings between embryos while maintaining normal polarity (sham controls).

HRP tracing method

At 10 or 11 days of incubation (stage 35–36), the embryo was bled, eviscerated and the brachial spinal cord exposed ventrally. The brachial and thoracic region were pinned out in a dish containing oxygenated Tyrode solution.

The operated and control limbs (still attached to the trunk) were sketched, viewed from the dorsal aspect. The level of the rotation (normally shoulder or elbow) was determined from the pattern of the feather germs and from the general

surface anatomy. A superficial dissection was performed distal to the plane of rotation on both operated and control sides to expose either the flexor or extensor muscles. The muscles on the operated side were rarely as well developed as on the control side and sometimes were puny. One of the better developed muscles in the operated limb together with its contralateral homologue were injected with up to 1μl concentrated (approximately 20 % w/v) aqueous horseradish peroxidase (HRP, Sigma type VI). The pipette was usually inserted into the muscle from the distal end and passed within the belly of the muscle to the origin; HRP was ejected as the pipette was withdrawn. This procedure was usually repeated three or four times without withdrawing the pipette from the original point of insertion, but in some cases only one pass was made.

The specimens were left in the oxygenated Tyrode on a gentle shaker at 38 °C for about 6 h when they were trimmed and fixed by immersion in cold 2 % gluteraldehyde in 0·1 M phosphate buffer at pH 7·4 for 3 h. The fixed tissue was transferred through several changes of 0·1 M phosphate buffer with 20 % sucrose and left in this overnight. The specimens were placed in gelatin-albumen for 1 h before being embedded in the same mixture. Alternate sections (unless otherwise stated) were cut in the transverse plane at 60 μm and mounted onto gelatinized slides which, after drying, were reacted with benzidine dihydrochloride according to the method of Mesulam (1976). Sections were lightly counterstained with neutral red before being quickly dehydrated and mounted in XAM (Gurr).

Camera-lucida drawings were made of every second section and the position of the labelled cells marked.

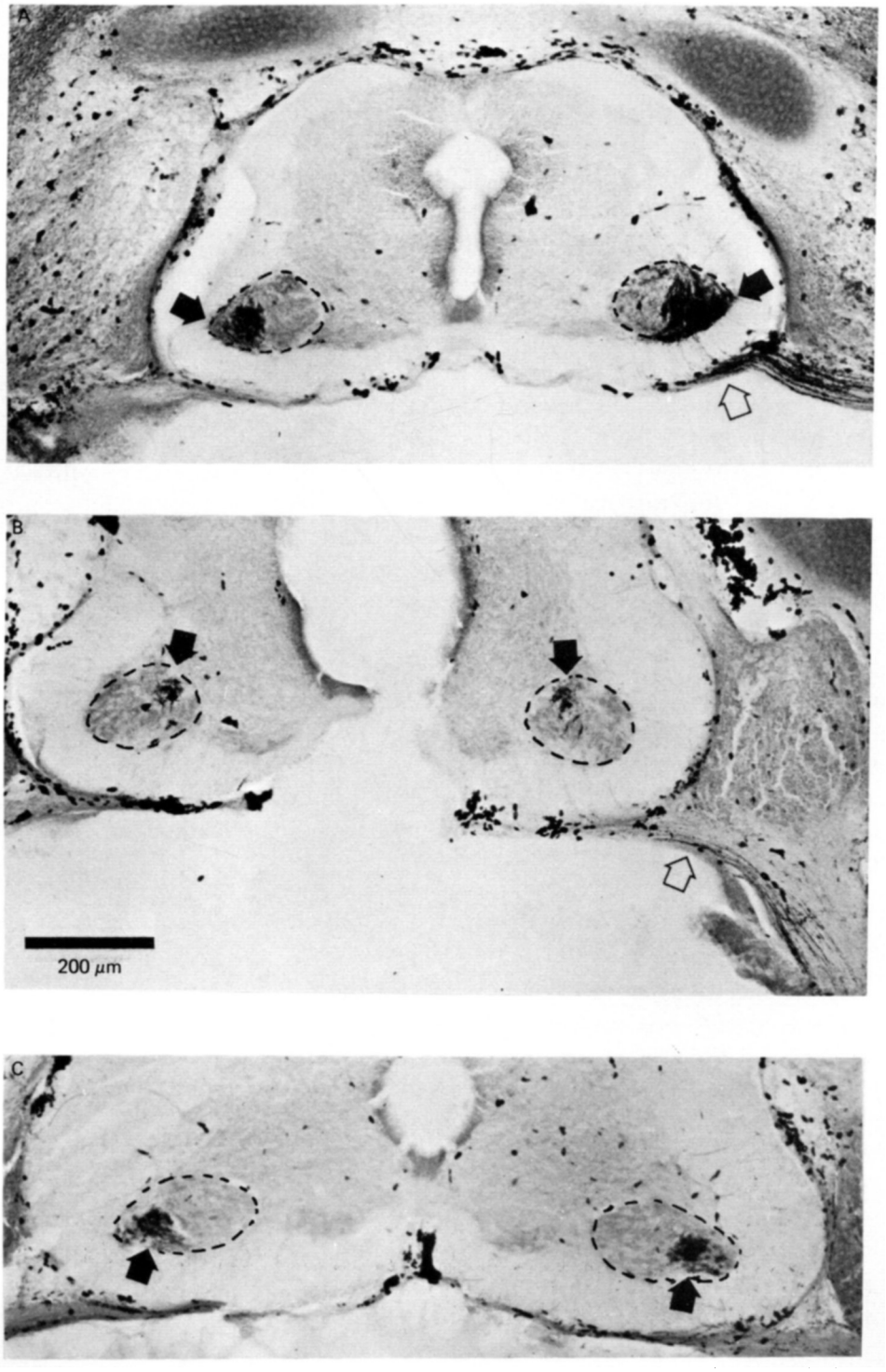
Some embryos were not used for HRP injection but the limbs were fixed and stained as whole mounts using the modified Bodian method developed by Lewis (1978).

RESULTS

Gross anatomy

Minor variations in the exact level of incision meant that the level of reversal was not always precisely at the shoulder or elbow. Following reversals at the more distal level the details of surface anatomy, particularly the feather germs, marked the change of axis distal to the cut. Rotations at the more proximal levels were rather more variable. This probably reflected the added difficulty of operating at the earlier stages, when the bud was significantly smaller and slight errors in positioning were more magnified during subsequent growth. The general appearance of the rotation was not as easily related to the operation as in the case of elbow rotation. This was because normally the wing had folded across the back instead of across the chest so that the ventral surface of the wing lay against the dorsal surface of the host.

Examination of whole mounts of Bodian-stained limbs showed that the anatomy of the nervous system proximal to the plane of section was always


A
B
200 μm
C

within the normal limits of variation of an unoperated control population. Distal to the plane of section, the majority of limbs showed a pattern of nerves that conformed to the tissue that they traversed, i.e. they too appeared dorsoventrally inverted. Thus a normal radial nerve passing the host/graft junction thereafter behaved as a medio-ulnar nerve, and *vice versa*. In this majority group there were on occasions minor abnormalities, particularly near the host/graft junctions. Sometimes the main nerves divided into several separate branches to rejoin further distally. The sham-operated controls had a nerve pattern within the normal limits of variation of the unoperated controls.

HRP labelling

The method used gave a dark blue granular reaction product in motor neurones in the ventral horn, which could be clearly distinguished from the reaction of endogenous peroxidase in red blood cells (Fig. 1). The neutral red counterstain showed clearly the anatomy of the cord and the surrounding tissues so that the boundary of the motor horn was clearly visible. Between segments 13 and 17 the enlargements of the motor horns and the very obvious dorsal root ganglia allowed one to determine unambiguously the segmental level of each section.

The reaction product in individual cells varied from very dense, where the soma and proximal dendrites were filled, to a light and scattered granular labelling confined to the soma (Figs 2 and 3). We have as yet been unable to determine the cause of this variation. Normally most of the label lay within a cluster or column of cells within the motor horn, but lightly labelled cells often lay outside the main cluster. Bilaterally symmetrical injections in control animals labelled cells in the same locality on the two sides, so that each muscle pool had its characteristic position (Fig. 1). All our analysis is based on identifying the position of the main cluster.

Rather than map the whole wing we have concentrated on two extensors derived from the dorsal muscle mass and two flexors from the ventral muscle mass (Sullivan, 1962). We chose the main flexor biceps (Bi) and the main extensor triceps (Tri), in the upper arm; and flexor carpi ulnaris (FCU) and extensor metacarpi ulnaris (EMU), in the forearm. In all cases one of us chose and injected the muscles while the other prepared the sections and mapped the position of the labelled cells.

Fig. 1. Symmetrical labelling of cells following (A) bilateral injection into triceps of a stage-30 unoperated embryo, axons filled with reaction product (open arrow) exit the cord laterally from the lateral extensor pool (solid arrow) to the 15th segmental root. (B) bilateral injection into biceps of a stage-35 unoperated embryo. Axons from the dorsomedial flexor pool (solid arrow) run ventrally through the white matter to lie ventrally in the root. (C) bilateral injection into triceps of a stage-35 sham control embryo, with operated limb on right. RBC: red blood cells. Dotted line marks boundary of motor horn.

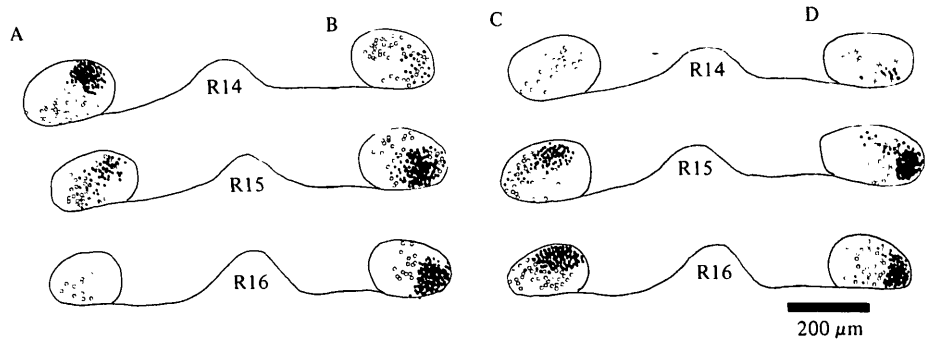


Fig. 2. A composite diagram taken from camera-lucida drawings of sections of normal ventral horn in stage-35 embryos. The positions of heavily (solid circles) and lightly (open circles) labelled cells at the level of the 14th, 15th and 16th segmental roots are shown following injection into (A) biceps, (B) triceps, (C) flexor carpali ulnaris (D) extensor metacarpi ulnaris. The labelled cells in a single section at each level from twelve cases have been superimposed.

Normal limbs

We mapped the pools to each of the four muscles following bilaterally symmetrical injections to each muscle in normal animals at stage 35. There were minor variations in the pool sizes and their rostrocaudal extent, but the position of the most densely labelled column of cells was always symmetrical. Despite variations in labelling density the pool positions were remarkably constant. In every case the person preparing the motor pool map correctly identified the injected muscle.

Figure 2 shows a composite map based on camera-lucida drawings. The flexor muscles are supplied by motoneurones situated dorsomedially, while the extensor muscle pool is more ventral and lateral in the motor horn. The pools also have a definite segmental arrangement within the cord, distal muscle pools lying more caudally than those supplying more proximal muscles.

A second smaller series of injections into muscles of stage-30 embryos showed an essentially similar pattern of pool positions. This stage also gave an improved chance of finding labelled motor axons. Axons from motoneurones labelled after flexor muscle injection, ran ventrally in the horn and left the cord lying more ventrally in the motor root than the axons from the extensor muscle pool which leave the horn more laterally (Figs 1 and 6). We did not usually inject animals at this stage since the muscles are much smaller than at stage 35, so that it was difficult to be sure of a good injection confined to the desired muscle, especially in operated limbs.

As a control experiment we made comparatively large injections under the skin above either a muscle or the radial nerve where it passes close to the surface near the elbow. This never resulted in a column of labelled cells in the ventral horn though sometimes occasional scattered labelled cells were seen.

Table 1. *Position of labelled cells in experimental wings*

Muscle injected	Total	Motor pool in position of antagonist	Motor pool in normal position	Position of motor pool undecided
Triceps	8	8 (100%)	0	0
Biceps	15	9 (60%)	5 (33%)	1 (7%)
EMU	23	12 (52%)	6 (26%)	5 (22%)
EMR	4	3 (75%)	0	1 (25%)
FCU	17	7 (41%)	8 (47%)	2 (12%)

We concentrated on situations which did not give consistent change in pool position.

Reversed limbs

A total of 75 embryos survived to ten days and had limbs of normal appearance save that the operated limbs were clearly reversed. The operated wings were normally slightly smaller than the contralateral controls, and the muscles were not quite as well developed. In some cases it was difficult to find particular muscles, so we injected the best developed. We used the same set of muscles as described for normal limbs but included in addition a few cases of extensor metacarpi radialis (EMR). Normally the same muscle was injected on both operated and control sides of the embryo so that it was easy to see if the pool positions were different. In other cases antagonistic muscles were injected, mainly as a means of testing the single blind system of mapping and identification of pool position. In eight embryos (11 %) we were unable to detect labelled cells on the operated side (this compares with 4 % for control sides). These embryos have not been included in the table of results. Of the remaining 67 embryos (see Table 1), in a minority of cases the labelled cells occupied positions appropriate to the muscle injected, in the majority, the cells occupied positions appropriate to the antagonist of the muscle injected. The remainder had labelled cells in both medial and lateral regions of the horn, i.e. appropriate both to the muscle injected and to its antagonist. The exception was FCU where half the cases showed preservation of pool position.

Figure 3 shows examples of reversed pool position following bilateral injections of triceps, biceps, flexor carpi ulnaris and extensor metacarpi ulnaris. Figure 4 shows examples of symmetrical pool position following bilateral injection into biceps, flexor carpi ulnaris and extensor metacarpi ulnaris. As seen in the table, injection into triceps muscle of the rotated wing always labelled medial cells in the horn. Note that on both operated and control sides weakly-labelled cells are sometimes found in other regions of the horn.

In those cases where biceps and triceps pools occupied reversed mediolateral position, we also found a change in their rostrocaudal position. Normally biceps is innervated by segments 14 and 15 while triceps is innervated by segments 15 and 16. In these operated cases the biceps pool occupied more caudal segments

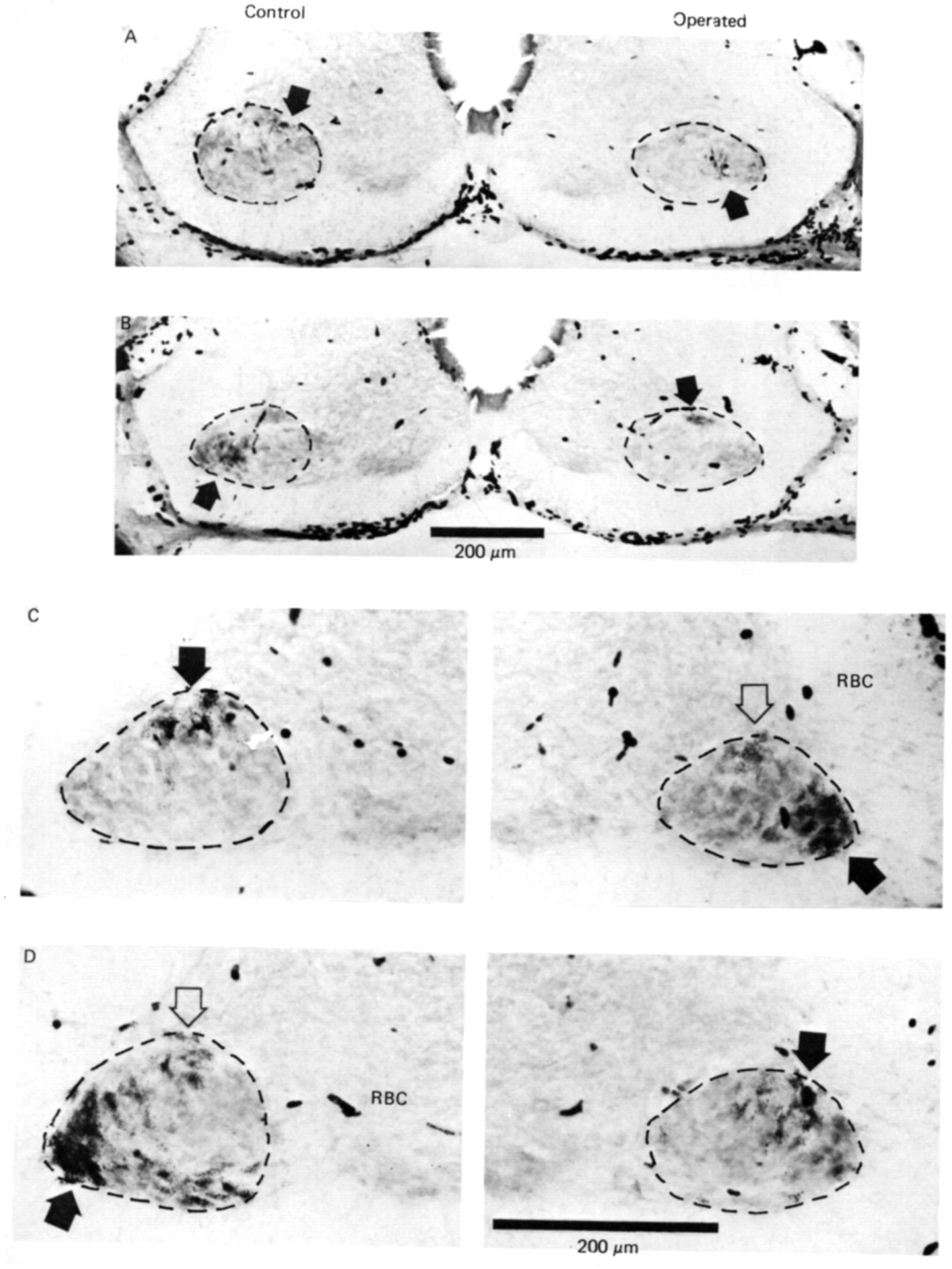


Fig. 3. Asymmetrical labelling of cells following bilateral injection in operated animals (A) biceps, (B) triceps, (C) flexor carpi ulnaris, (D) extensor metacarpi ulnaris. The control side is shown to the left with the reversed wing to the right. The main position of the cells on the operated side occupy the position characteristic of the antagonist of the injected muscle. Solid arrows indicate densely labelled cells, open arrows weakly labelled cells. Dotted line marks boundary of motor horn.

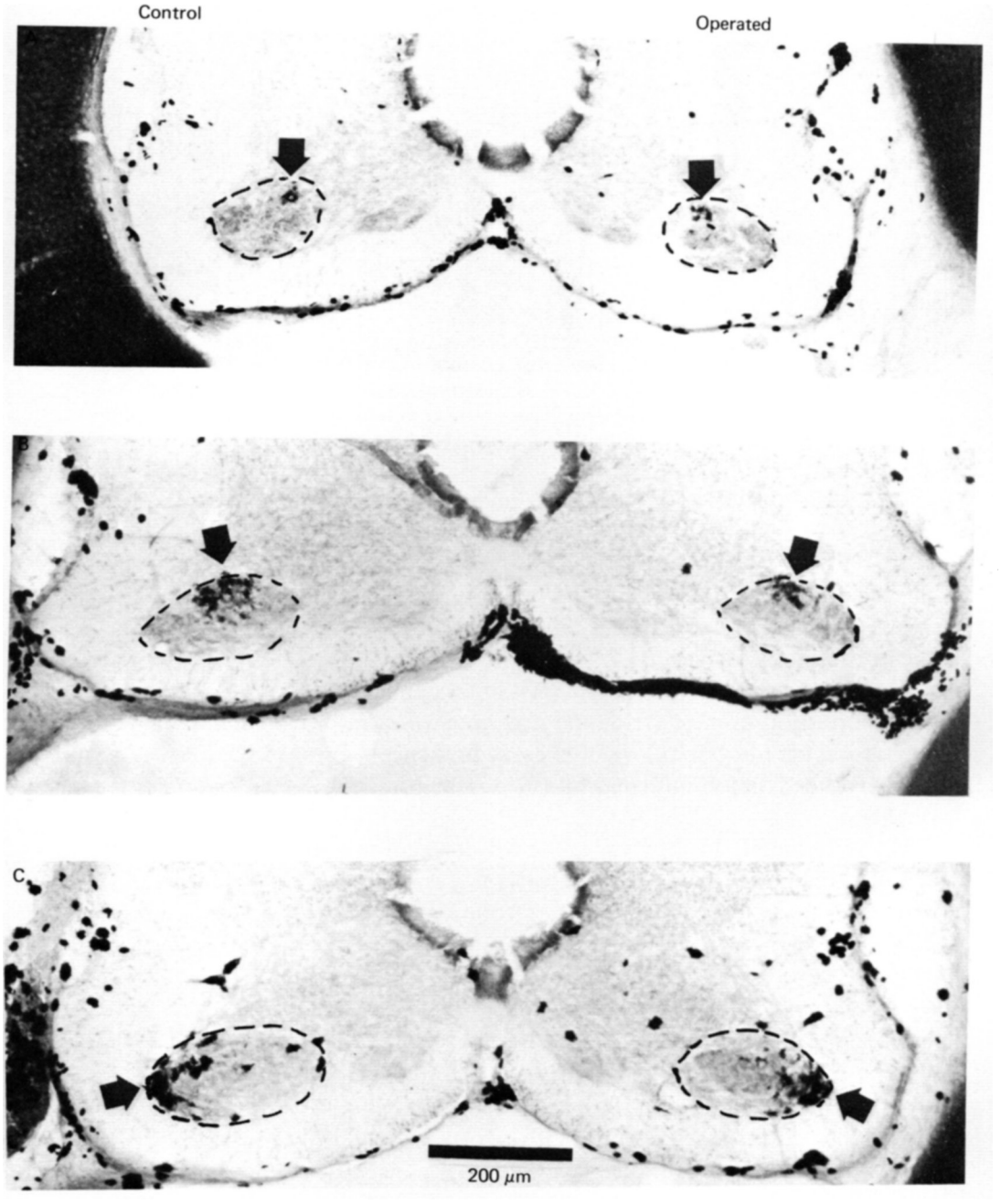


Fig. 4. Three cases where bilateral injection of (A) biceps, (B) flexor carpi ulnaris, (C) extensor metacarpi ulnaris, resulted in labelling of clusters of cells (arrowed) occupying symmetrical positions on the operated and control sides (triceps injections in operated animals never gave symmetrical labelling). Dotted line marks boundary of motor horn.

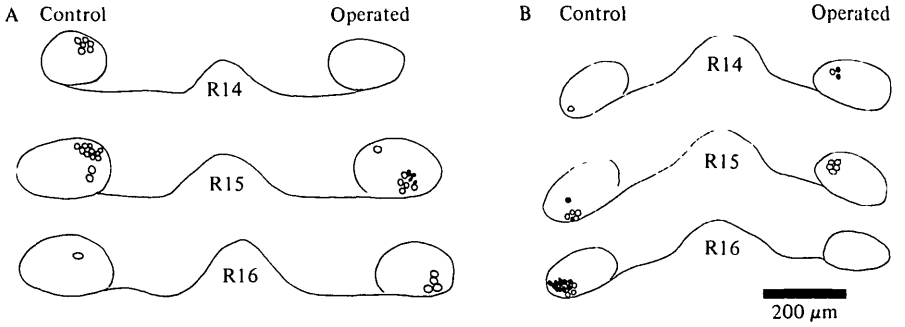


Fig. 5. Camera-lucida drawings of single sections of the motor horn at the level of 14th, 15th and 16th spinal roots in two operated animals. (A) Bilateral injection into biceps. The labelled cells on the operated side occupy a more caudal and lateral position than on the control side. (B) Bilateral injection into the triceps muscle. The labelled cells on the operated side occupy a more anterior and medial position than on the control side.

and the triceps pool more rostral segments. This is illustrated by the camera-lucida drawings in Fig. 5. A hint of a similar change was seen in a few of the rotated limbs in which more distal muscles had been injected. The limbs showing this change in position always had flexor muscles innervated by lateral pools or extensor muscles innervated by medial pools, and never had labelled cells in the appropriate position.

Two factors which must play a part in this phenomenon are the arrangement and proportion of axons from the segmental roots in the radial and medioulnar nerves and the position of origin of nerve branches to biceps and triceps. Because we have no detailed information on this at present we shall not discuss this further.

We were unable to discover any clear correlation between the three types of result in the table and any of the variables in our experimental methods (stage of operating, proximodistal level of rotation, external appearance of rotated limb).

Sham controls

A total of 12 embryos survived to day 10 and had limbs of normal appearance save that the operated wing was sometimes smaller than the contralateral control and sometimes had minor abnormalities proximally (presumably at the plane of incision). In most cases we injected the triceps bilaterally because this was the muscle that in reversed limbs gave the most consistent (100 %) pattern having the motor pool in the position of the antagonist (Table 1), but we did perform some injections in each of our usual muscle range. In all cases the position of the labelled motor pools were bilaterally symmetrical (Fig. 1*c*).

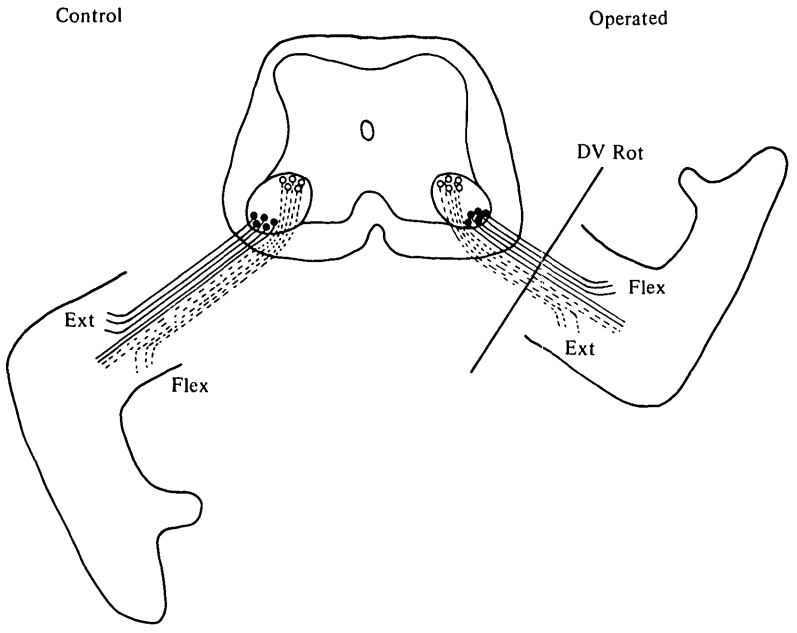


Fig. 6. Diagram of cells and their axons supplying the flexors (solid circles) and extensors (open circles) in control (to the left) and rotated (to the right) wings. The positions of the axons in the normal wing are based on specimens such as shown in Fig. 1, while those in the rotated wing show the trajectories expected on a passive rather than active guidance model (see text).

DISCUSSION

The experiments described in this paper were designed to discriminate between three models for the development of innervation pattern. We consider here how well the data fit each of the three theories introduced at the start of the paper.

Passive segregation

Our own working hypothesis (Stirling & Summerbell, 1979) supposes that innervation is based on loosely defined tracts running through the tissue, a system of environmental cues that map out the positions of future nerves. Invading axons explore the area ahead and advance down any of these 'nerve-tracts' that they encounter. Axons tend to maintain their positions relative to one another, not normally crossing, but following the path of any pre-existing axon that they encounter, and always run distally. When a branch point is en-enountered in a tract the branch taken by a particular axon depends on its position within the tract, there is no 'seeking' of the correct tract, axons pass down the nearest branch. Axons from neighbouring neurones (and therefore a particular part of the cord) tend to reach the same general target area (therefore showing apparent specificity) having traversed the same route (therefore having the same segmental origin). During normal development an axon's target is a function of its position in the cord.

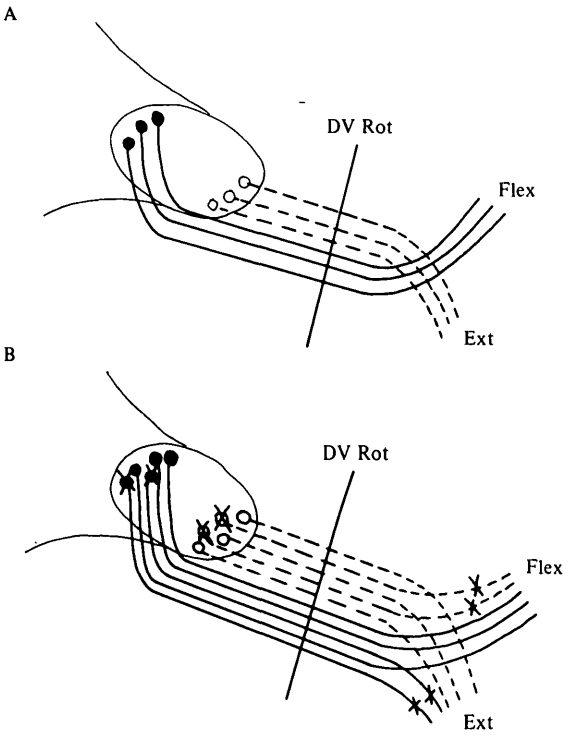


Fig. 7. Diagrammatic illustration of the distribution of motor pools to flexors (solid circles) and extensors (open circles) and their axons (solid and dotted lines respectively) to be expected following limb rotation according to A, the specificity of axon outgrowth model and B, the selective cell death model. In B those cells and axons which have contacted inappropriate targets and are destined to die are marked with a cross.

This hypothesis makes firm predictions about the experiments described in this paper. As the axons exit the motor horn ventrally then set off in the direction of the flank, the only arrangement that gives ordering without major crossing of axons is for the axons of medial origin to lie ventral to the axons of lateral origin in the spinal root (Fig. 6). This relative arrangement is maintained through the plexus so that the axons of medial origin enter ventral nerves and innervate flexor muscles. Axons of lateral origin enter dorsal nerves and innervate extensor muscles. When the limb is reversed about its dorsoventral axis prior to innervation, the main dorsal nerves find themselves confronted by ventral limb tissue and *vice versa*. The dorsal nerve continues to follow positional cues distally down the limb, enters the ventral nerve tract of the graft and innervates ventral targets.

This hypothesis therefore predicts that HRP injected into a muscle of the dorso-ventrally rotated graft should label motoneurones in the position appropriate to its antagonist. The prediction was fulfilled in the majority of cases. The proportion of successful reversals varied from 100 % for triceps to 41 % for

FCU. We would not expect to find cases in which there were labelled neurones in the position appropriate to the muscle injected.

Active selection

This hypothesis (see, for example, Landmesser & Morris, 1975) supposes that the cell bodies in the motor horn are initially specified with respect to their peripheral destination and that nerve outgrowth is selective. The axons reject unauthorized tracts and use the positional cues in the limb to seek out their proper pathways from the start. It predicts that HRP injected into a muscle of the dorsoventrally rotated graft should label motoneurones in the muscle's normal pool (Fig. 7A).

The predictions of this hypothesis were fulfilled in a minority of our cases (varying from 47 % FCU to 0 % triceps). If one includes undecided cases (where axons may have had difficulty in reaching their target, allowable under this hypothesis) then they still constitute a minority for most muscles (59 % FCU, to 0 % triceps (see Table 1).

This hypothesis does not predict the labelled cells found in the position appropriate to the antagonist of the muscle injected.

Random outgrowth and cell death

This hypothesis (see, for example, Pettigrew *et al.*, 1979) again supposes that the cells in the cord are specified with respect to their peripheral destination. However the axons grow out randomly so that some axons from every part of the horn reach muscles in every part of the limb. Neurones inappropriately connected subsequently disappear during the period of cataclysmic cell death between stages 31 and 35. This hypothesis predicts that by stage 35 there should be no inappropriately labelled cells but that all muscles should be correctly connected (Fig. 7B). This was again fulfilled only in a minority of cases, the figures being the same as for the preceding hypothesis.

A reconsideration of models

Clearly the data does not provide unequivocal support for our earlier working hypothesis (Stirling & Summerbell, 1979), nor is it encouraging for either of the two alternatives. In the majority of cases the axons have innervated 'inappropriate' muscles in a way that is explained parsimoniously by passive deployment but in a large minority the apparent selective behaviour supports the other two hypotheses.

The existence of cases with inappropriate motor connexions is most difficult to explain with the random innervation and selective cell death hypothesis. A possible solution that retains some of the features of the latter idea is suggested to us by the Tea-Trade model of Malsburg & Willshaw (1977), (which is concerned with the formation of ordered connexions between retina and optic

tectum). We again assume passive segregation but with a progressive deterioration of ordering as one proceeds distally (Sunderland & Ray, 1948) so that axons from a particular origin in the cord have only a certain probability of arriving in a particular muscle. There is then an interaction between neighbouring nerve and muscle that tends to stabilise neurones with many neighbours terminating in the same muscle, but tends to eliminate (by cell death) neurones with few neighbours. The 'dominant' pool promotes its own position at the expense of more scattered neurons. This idea needs to be explored further by simulation to determine if plausible results can be obtained, it may be necessary to include a weak starting advantage for 'appropriate' neurones.

A simpler explanation of the results is provided by a fusion of the active selection and passive segregation hypotheses. We again assume the passive segregation rules for axonal outgrowth but add a weak short-range selective mechanism influencing the axon's interaction with the environment. In the normal embryo axons grow out into the limb and because of the passive segregation rules tend to reach branch points in such a position in the main trunk that they normally turn off to their correct targets. Random errors in the position of an axon near a branch point can be corrected by a short-range selective signalling mechanism so that the axon diverts into the appropriate route.

Following dorsoventral rotation of the limb the axons are artificially diverted down an incorrect route. They will therefore not normally encounter any environmental cues that they recognize as the indicators for the 'appropriate' target. They therefore obey only the passive deployment rules and produce reversed innervation (e.g. triceps?). However in a small innervation field such as the limb, there are likely to be circumstances in which different nerve tracts closely approach each other. It is possible that the axons then come within range of cues that they can recognise as being specific to their target (e.g. FCU?). They can then be diverted into their appropriate final paths. It is interesting that Landmesser & Lance-Jones (personal communication) have evidence that axons are able to correct for small rotations of the spinal cord but are unable to do so for large ones.

CONCLUSION

If there be an active mechanism attempting to match motoneurones to their 'appropriate' muscles it cannot be very effective. Nevertheless we cannot exclude very short-range signals. Our next step is to correlate the detailed anatomy of the nerves in operated limbs with the pattern of pool labelling in cord. The trajectories of axons from segmental nerves into reversed limbs and from injected muscles to the cord should further illuminate our understanding of the factors responsible for the generation of the innervation pattern.

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