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THE DEVELOPMENT OF SENSORY PROJECTION PATTERNS IN EMBRYONIC CHICK HIND LIMB

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SUMMARY

1. The distribution within individual dorsal root ganglia (d.r.g.s) of sensory neurones projecting to different targets in the embryonic chick hind limb was determined using the retrograde transport of horseradish peroxidase (HRP). The segmental pattern of sensory neurone projections was also defined, using retrograde and orthograde HRP labelling and electrophysiological techniques, from the onset of axonal outgrowth into the limb until after the period of sensory cell death.

2. At stage (St.) 29–30, shortly after initial axonal outgrowth into the limb, the large lateroventral neurones in the d.r.g.s projected both to skin and to muscle. At St. 36–37, after cell death in the d.r.g.s, cells from both the lateroventral and mediodorsal populations projected both to skin and to muscle. Thus these two cell populations do not correspond to cutaneous and proprioceptive afferents, respectively.

3. The cells projecting along individual cutaneous nerves or to individual muscles were always widely distributed throughout the ganglia. Thus, sensory neurones cannot be specified to project to particular peripheral targets as a result of their position in the d.r.g. Nevertheless, small clusters of cells were frequently found to project along the same peripheral nerve.

4. Since there is a correlation between position in the d.r.g. and time of origin, neurones projecting to each target have a wide range of birthdates, and, therefore, sensory neurones cannot be specified as a result of their birthdate.

5. At St. 36–38, after cell death, afferents to a given muscle or cutaneous nerve arise primarily from two or three adjacent segments out of the eight lumbosacral segments. For muscles these are the same segments that supply the motoneurones to that muscle. Each d.r.g. sends a characteristic proportion of axons down each of several peripheral nerves in a consistent and orderly pattern.

6. During initial outgrowth, the segmental projection pattern is similar to the pattern found in mature embryos. Thus, extensive projection 'errors' are not made and neither cell death nor retraction of axons is necessary for establishing the appropriate connectivity pattern. The majority of neurones do not send branches down more than one peripheral nerve.

7. Axons projecting to the same target are initially dispersed in the spinal nerves, and gradually segregate out in the plexus region, ultimately to form a separate nerve trunk. Axons projecting to different targets cross each other. Cutaneous and muscle

nerves first form at the same stages. Therefore, the particular pathways axons take do not depend in any simple way on either axonal position in the plexus or time of arrival at the base of the limb. Simple timed outgrowth mechanisms and models in which axons maintain constant topographical relationships with each other therefore cannot generate the observed projection pattern.

8. The questions of whether sensory neurones are specified prior to outgrowth, whether afferents actively choose certain pathways or are passively channelled into them, and whether motoneurones might play a role in guiding afferent outgrowth are discussed.

INTRODUCTION

It has frequently been suggested that neural crest cells initially are pluripotent (Weston & Butler, 1966; LeDouarin & Teillet, 1974; LeDouarin, Renaud, Teillet & LeDouarin, 1975; Noden, 1975) and that the course of their subsequent cytodifferentiation is determined by interactions with their environment (Cohen, 1972; LeDouarin & Teillet, 1974; LeDouarin *et al.* 1975; Noden, 1978*a*, *b*; but see Cohen, 1977). Since these interactions may affect not only cell type (e.g. melanocyte *vs.* neurone) but also such characteristics as neuronal morphology and transmitter metabolism (see Patterson, 1978 for review of tissue culture studies), the question of whether these interactions influence another neuronal characteristic, the specification of peripheral projections, should be considered. Are crest-derived neurones specified for their targets before, during or after migration, or do they become specified only after contact with the targets themselves? For instance, it has frequently been proposed (for reviews see Gaze, 1970; Jacobson, 1978; Baker, 1978), particularly for cutaneous afferents on the basis of skin-rotation experiments in frogs, that the periphery may determine a neurone's central connexions. However, it is not possible to determine whether a population of neurones is unspecified or specified nor to elucidate the mechanism by which appropriate projections are attained unless one is able to identify cells which project to different targets, by some marker independent of the target, for instance the cell's position. With positional markers one can ascertain whether cells project to their appropriate targets initially during development as well as after various experimental manipulations.

The possibility that there might be a correlation between the position of the cell body in the dorsal root ganglion (d.r.g.) and the peripheral target site was suggested by the presence of two morphologically different cell types localized in distinct regions of the d.r.g. in 7–15-day-old chick embryos. The large, early differentiating cells in the lateroventral region were presumed to be cutaneous afferents based on the early onset of cutaneous reflexes, whereas the small, later differentiating cells in the mediodorsal region were presumed to be proprioceptive based on the later appearance of proprioceptive reflexes (Hamburger & Levi-Montalcini, 1949).

An alternative or additional possibility was that a consistent and specific segmental pattern of projections could be defined. Previous results (Landmesser & Morris, 1975) had implied that the afferents to individual muscles originate from the same restricted subset of spinal nerves as do the motoneurones.

In the present study, the retrograde transport of horseradish peroxidase (HRP) was used to determine the distribution of afferent cell bodies within individual ganglia projecting to different targets in the limb and, in conjunction with orthograde labelling and electrophysiological techniques, to define the segmental patterns of sensory neurone projections. The initial outgrowth of afferents into the limb has been examined to determine if the initial sensory innervation of muscles is selective, as suggested by previous experiments (Landmesser & Morris, 1975; Landmesser, 1978*b*) or diffuse and random (as suggested by Pettigrew, Lindeman & Bennett, 1979, for motoneurones in the chick wing, but see Oppenheim, 1981). The outgrowth of afferents projecting to the skin has also been studied. Finally, this description of the initial and mature patterns of afferent projections forms a necessary background for a comparison of experimentally manipulated embryos (Honig, 1979; M. G. Honig, C. Lance-Jones & L. Landmesser, in preparation).

Preliminary reports of these results have been presented elsewhere (Honig, 1977, 1979).

METHODS

General procedure

White Leghorn chick embryos were incubated in a forced draft incubator until Stage (St.) 24–38 (Hamburger & Hamilton, 1951). Embryos were decapitated, eviscerated, the skin removed and a ventral laminectomy performed, exposing the spinal cord from high thoracic to lumbosacral levels. Experiments were performed on this isolated spinal cord–hind limb preparation in a dish containing oxygenated Tyrode (139 mM-NaCl, 3 mM-KCl, 17 mM-$NaHCO_3$, 3 mM-$CaCl_2$, 1 mM-$MgCl_2$, 0·22% dextrose, pH 7·2–7·3) solution.

Application of horseradish peroxidase

Retrograde labelling with HRP was used to localize afferents projecting to several target sites in the limb in St. 35–37 embryos. Targets were chosen to allow the comparison of cell body localization of different types of afferents within single ganglia. Axons to the three muscles studied, the sartorius, femorotibialis and adductor, originate from lumbosacral (LS) spinal nerves 1–3 (corresponding to segments 23–25) which first converge in the crural plexus (Fig. 1). The lateral femoral cutaneous nerve and the medial femoral cutaneous nerve (Getty, 1977) also originate from LS1–3. The lateral femoral cutaneous nerve exits from the thigh between the sartorius and iliotibialis to innervate the pre-axial surface of the thigh. The medial femoral cutaneous or saphenous nerve runs along the medial surface of the thigh between the femorotibialis and adductor muscles and innervates the skin overlying the knee and shank.

Injection of muscles with HRP (Sigma Type VI) was performed as described by Landmesser (1978*a*). A cutaneous nerve was filled by cutting it distal to where it emerges from the limb musculature and sucking the proximal cut end up into a suction electrode containing HRP 50–100 mg/ml. Exposure of the nerve to HRP continued for $\frac{1}{2}$–2 h.

In St. 29–30 embryos, cutaneous nerves were filled in the same manner described for older embryos. Injections were made into one of the two primary muscle masses of the thigh as described by Landmesser (1978*b*) except that the overlying skin was removed to prevent the spread of HRP to nerve endings in the skin. Injections were also restricted to part of the muscle masses: the region of the prospective adductor and ischioflexorius muscles for the ventral muscle mass of the thigh and primarily the medial and anterior parts of the prospective sartorius, femorotibialis and anterior iliotibialis for the dorsal muscle mass. In addition, embryos that had received HRP injections into local regions of the ventral muscle mass at St. 28 and of the adductor at St. 30–34 for a previous analysis of motoneurone projections (Landmesser, 1978*b*) were examined to determine the distribution of labelled afferents. Injection sites in St. 28–34 embryos were often verified by examination of sections through the limb after histological processing.

Histological processing

After injection, specimens were incubated in oxygenated Tyrode solution at 32–35 °C for at least 5 h to allow retrograde transport of HRP. Spinal cords and adjacent ganglia were then fixed in 2% glutaraldehyde and processed using diaminobenzidine, as previously described (Landmesser, 1978*a*). Paraffin-embedded sections were cut transverse to the long axis of the spinal cord. The sections were generally 10 μm thick except in the initial series of St. 29–30 embryos which were 14 μm thick.

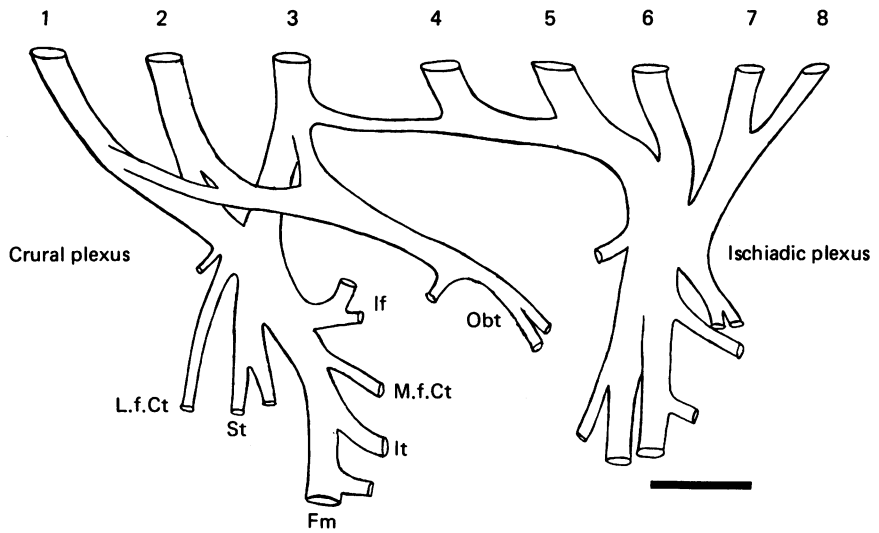

Fig. 1. Schematic diagram of the innervation of the chick hind limb based on camera lucida drawings of cross-sections through the limb of a St. 29 embryo. Lumbosacral spinal nerves 1–3 form the crural plexus; the occasional contribution of the last thoracic spinal nerve, T7, to the crural plexus is not shown. Spinal nerves 4–8 with a contribution from spinal nerve 3 form the ischiadic plexus. Several peripheral nerves are shown. These include the lateral femoral cutaneous (L.f. Ct) and the medial femoral cutaneous (M.f. Ct) nerves. The sartorius (St), femorotibialis (Fm), iliotibialis (It) and iliofemoralis (If) muscle nerves are also shown. The obturator (Obt) muscle nerve innervates both the obturator and adductor muscles. Calibration bar = 200 μm.

The sections were counter-stained with cresyl violet. Cells that showed labelled cytoplasm at least partly surrounding an unlabelled nucleus were counted, using a Zeiss compound microscope at a magnification of 790–1260 ×. Pycnotic labelled cells were not counted. In general d.r.g. cells in St. 36 embryos contained a granular reaction product whereas in St. 29–30 embryos a combination of diffusely as well as granularly labelled cells was found. The type of reaction product cannot therefore simply be related to damage since the cutaneous axons were cut at both stages.

Following injection of individual muscles, labelled motoneurones were counted and histograms showing the number of labelled cells in two to three adjacent sections were drawn to compare their distributions with those from a previous study (Landmesser, 1978*a*). Following cutaneous nerve fills, the lateral motor column was examined to verify the absence of labelled motoneurones. These controls insured that the injections filled fairly completely but did not spread beyond the desired regions.

Labelled d.r.g. cells were counted in every section at a magnification of 790–1260 ×. Camera lucida drawings, at a magnification of 200–320 ×, of spinal cord, d.r.g. and labelled cells were made of every section for St. 29–30 embryos and of approximately every third section for St. 36 embryos. These drawings were superimposed, using the outline of the spinal cord as a reference point, so that each drawing shows the position of labelled cells throughout the rostral-caudal extent of the

ganglion. The outlines of the d.r.g.s were obtained by drawing around all the superimposed traces and represent the greatest extent of each d.r.g. in the ventral-dorsal and medial-lateral axes.

Quantification of incidence of clusters of labelled cells

To quantify the frequency with which labelled cells were situated adjacent to each other, the number of labelled cells occurring in pairs and in larger clusters was counted separately to compare with the number of labelled cells occurring singly. Since no attempt was made to determine if a cell was adjacent to another labelled cell located in the next section, the number of pairs and clusters counted is probably an underestimate of their true number.

The percentage of labelled cells that might be expected to be adjacent to another labelled cell if labelled cells are randomly distributed in the d.r.g. was estimated in the following manner. If x = the total number of neurones in a d.r.g. and n = the number of labelled cells in that d.r.g., then the probability of any given cell being labelled is n/x. If each cell in the ganglion is adjacent to an average of y other neurones, the probability of a labelled cell being adjacent to another labelled cell is $y\ (n/x)^2$. Values of x and y were chosen to give the highest predicted probability. Individual neurones were usually adjacent to six other neurones in each section and only rarely were adjacent to more than eight, so a value of 8 was chosen for y. Since the total number of neurones in ganglia from embryos St. 35 and older ranged from 5100 to 15,800 (Levi-Montalcini & Hamburger, 1951; Mitolo, Selvaggi & Selvaggi, 1965; M. G. Honig, unpublished data), a value of 6000 was chosen for x for St. 35–36. In St. 29–30 embryos, since labelled cells were found in only the more mature lateroventral region of the ganglion (see Results) and the estimated number of differentiated large lateroventral neurones ranged from 2000 to 3500 (M. G. Honig, unpublished data), a value of 2000 was chosen for x.

Retrograde HRP labelling of axonal pathways in the limb

To follow the course of axons from their peripheral targets to the d.r.g.s or spinal cord, HRP was injected into either the lateral femoral cutaneous, medial femoral cutaneous or sartorius muscle nerves in a series of St. 29–32 embryos. After the usual reaction procedure, 10 μm thick sections were cut parallel to the longitudinal axis of the spinal cord. Axons labelled diffusely with HRP could readily be visualized in the peripheral nerve, plexus and spinal nerves. In some cases camera lucida drawings were made at a magnification of 125 × and labelling of axons confirmed at a magnification of 790–1260 ×.

Orthograde HRP labelling of axonal pathways

Individual d.r.g.s, exposed by cutting the overlying ventral roots, received multiple injections of 5–10 % HRP using a broken off micropipette. Damage to cell somas and/or neurites seemed necessary for uptake of HRP but, since afferents projecting to different targets are not localized within different regions of the d.r.g. (see Results), these fills did not have to label the entire ganglion to provide an adequate representation of the peripheral projections of the d.r.g. Diffusely labelled axons were traced into the limb in sections cut at 10 μm parallel to the longitudinal axis of the spinal cord. In some cases, the plexus, nerve branches and the courses of labelled and unlabelled axons were reconstructed from camera lucida drawings made at a magnification of 125 ×. The distal parts of the cut ventral roots were examined to check that HRP had not been picked up by motoneurone axons.

The numbers of labelled axons in the lateral femoral and medial femoral cutaneous nerves were estimated by examination of sections through these nerves under oil immersion at a magnification of 1260 ×. Labelled axons profiles with diameters of roughly 0·3–0·5 μm could be visualized, each of which probably represents just one, or at most three, unmyelinated axons, since axon diameters in 6½-day embryos show a unimodal peak at 0·5 μm (Verna & Saxod, 1979). Labelled axons running closely together were difficult to resolve individually and their numbers may have been slightly underestimated. The number of labelled axons in the two nerves was compared within individual preparations after injection of a given d.r.g. by assigning the total number of stained axons in both nerves a value of 100 % and expressing the number of stained axons in each nerve as a percentage of the total.

Electrophysiological experiments

St. 29–38 embryos dissected as described above, were hemisected and the spinal cord removed. The two most caudal thoracic (T6 and T7) and the eight lumbosacral (LS1–8) d.r.g.s were freed (see Landmesser & Morris, 1975) to allow spinal nerve stimulation. The two cutaneous, the obturator (which supplies the adductor and obturator muscles), and sartorius nerves were dissected free and cut. The preparation was maintained at room temperature (16–24 °C) in oxygenated Tyrode to which d-tubocurarine was added at high concentrations (5×10^{-5} M) to prevent the pick-up of electrical activity from muscles. Suction electrodes were used for stimulation and recording. Responses were recorded with either a Transidyne MPA-6 or Grass P15 differential amplifier and a Textronix 5030 oscilloscope and photographed with a Grass C4 camera.

Projection patterns were assessed in two ways. First, each of the spinal nerves was stimulated sequentially while recording from the cut proximal ends of the two cutaneous nerves. Responses were also recorded from muscle nerves, although the contributions of the motoneurone and sensory neurone responses to the compound action potentials could not then be distinguished. The area under each action potential response was measured. For each peripheral nerve all the responses were summated. The sum was assigned a value of 100% and responses to individual spinal nerve stimulation were then expressed as a percentage of this.

Secondly, recordings were made from the individual spinal nerves while successively stimulating each of the two cutaneous nerves to provide a comparison for a single spinal nerve of the relative numbers of axons projecting to these two nerves. The area under each compound action potential response was measured. For each spinal nerve, the response from each cutaneous nerve was expressed as a percentage of the sum of the responses from the two nerves.

No attempt was made to correct for the larger contributions to extracellularly recorded action potentials by larger, faster-conducting fibres in comparison to smaller, slower-conducting axons. Although this could bias the results in favour of the larger fibres, it should not affect the comparisons made here, since similar spectra of conduction velocity components were present from each spinal nerve to a given peripheral nerve and for the two cutaneous nerves (M. G. Honig, unpublished data).

To determine whether individual neurones send branches out more than one peripheral nerve, an attempt was made to record axon reflexes. One peripheral nerve was stimulated while recording from another peripheral nerve and sometimes while monitoring the response in one of the spinal nerves to ensure that stimulation was effective. Subsequently, the spinal nerves were stimulated and their responses summated as above, to provide a measure of the total compound action potential. The resolution of the peripheral nerve recording, estimated as the minimum response detectable above the noise level, was always 0·3–1·0% of the calculated total compound action potential.

Statistical analysis

The contributions of different spinal nerves to the same target, the same spinal nerve to different targets and the same spinal nerve to a given target at different stages were compared using the Wilcoxon rank sum test. A P value $\leqslant 0{\cdot}05$ was considered statistically significant.

RESULTS

The distribution of afferents within individual ganglia

Localization of cutaneous and proprioceptive afferents. The retrograde transport of HRP was used to determine the distribution within individual d.r.g.s of afferents projecting to muscle and to skin in St. 29–30 embryos, shortly after axonal outgrowth into the limb. Neurones projecting to the dorsal muscle mass, the ventral muscle mass and along the lateral femoral cutaneous nerve have similar distributions, as illustrated in Fig. 2. Labelled neurones in three cutaneous, three dorsal muscle mass and three ventral muscle mass fills were, in each case, widely distributed throughout the lateroventral part of d.r.g.s 1 and 2. The absence of labelled cells in the mediodorsal region is probably explained by the late birthdates of these cells (M. G. Honig, personal observation; M. McPheeters & L. M. Okun, personal communication; Carr

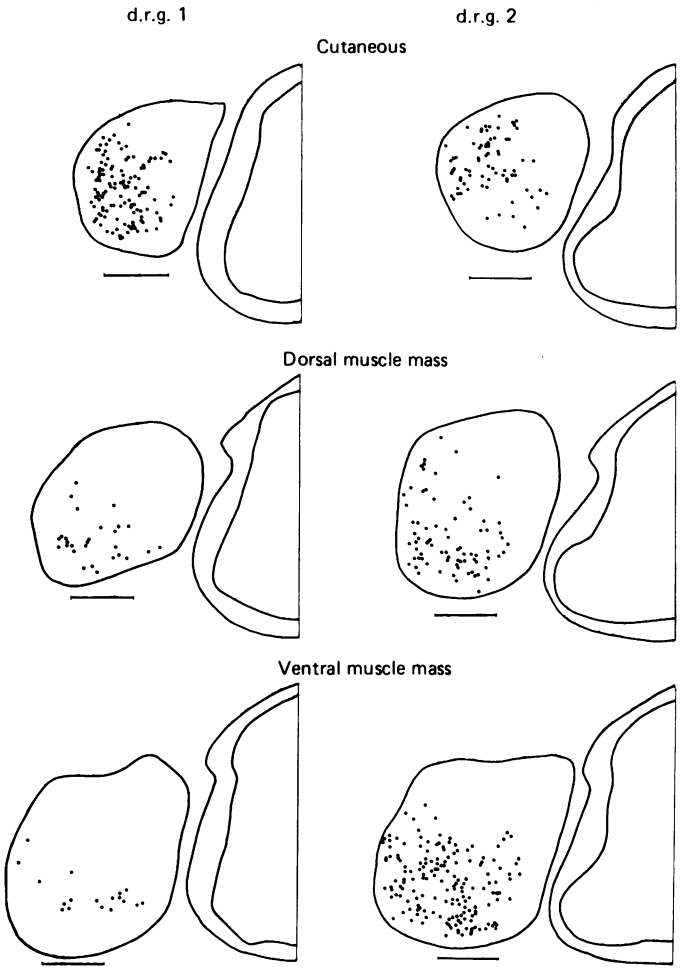

Fig. 2. Camera lucida reconstructions of lumbosacral d.r.g.s 1 (left) and 2 (right) showing the positions of cell bodies labelled by HRP fills of the lateral femoral cutaneous nerve (top), the dorsal muscle mass of the thigh (middle) and the ventral muscle mass of the thigh (bottom) at St. 29–30. Virtually all labelled cells are situated in the lateroventral half of each d.r.g. Traces were made of individual sections and then the drawings were superimposed. Each reconstruction shows all the labelled cells throughout the rostral-caudal extent of that d.r.g. in one embryo. The total number of labelled cells was, for the cutaneous fill, 123 in d.r.g. 1, 75 in d.r.g. 2; for the dorsal muscle mass, 28 in d.r.g. 1, 78 in d.r.g. 2; for the ventral muscle mass, 18 in d.r.g. 1, 171 in d.r.g. 2. Borders of spinal cords are shown for orientation: ventral is down, lateral is to the left. Calibration bar = 100 μm.

& Simpson, 1978*a*); their axons would have not yet entered the limb. Thus at St. 29–30 the large lateroventral cells project both to skin and to muscle, contrary to their previously assumed cutaneous projection (Hamburger & Levi-Montalcini, 1949).

It is possible that lateroventral cells might initially project randomly to both skin and muscle and that incorrect projections might subsequently be removed, perhaps

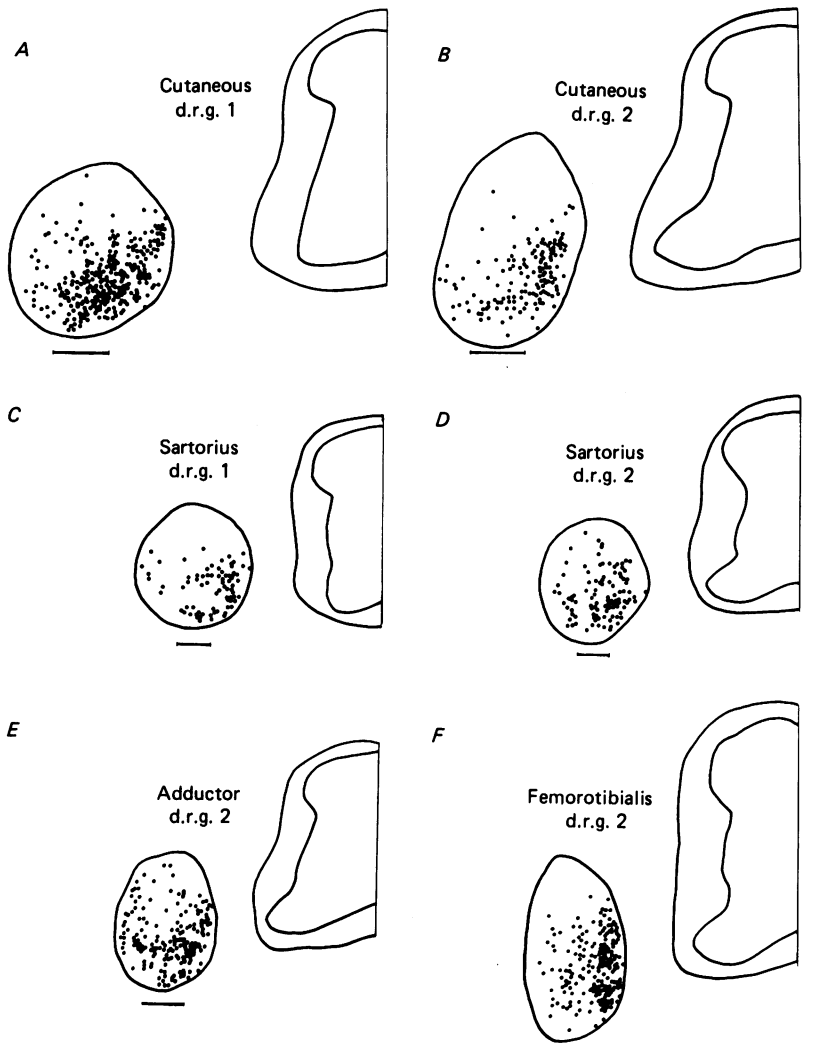

Fig. 3. Camera lucida reconstructions of d.r.g. 1 (*A*, *C*) or d.r.g. 2 (*B*, *D*, *E*, *F*) showing the positions of cell bodies labelled by HRP fills of the lateral femoral cutaneous nerve (top), the sartorius muscle (middle), the adductor muscle (bottom, left) and the femorotibialis muscle (bottom, right) in St. 36 embryos. The distribution of labelled cells was fairly widespread in all cases. Reconstructions were made as described for Fig. 2, but here show approximately one-third of the total number of labelled cells in each d.r.g. The total number of labelled cells was *A*, 782, *B*, 380, *C*, 168, *D*, 235, *E*, 373, *F*, 414. Calibration bar = 100 μm.

by cell death. Indeed, degenerating cells containing HRP were sometimes observed. To test this possibility, the localization of afferents was examined in a series of embryos at St. 36–37, after most cell death (Hamburger, Brunso-Bechtold & Yip, 1981). The distribution of labelled cells in d.r.g.s 1 and 2 was still fairly widespread in camera lucida reconstructions of three lateral femoral cutaneous and three sartorius fills, as illustrated by the one example of each shown in Fig. 3*A*–*D*, and also after filling

the medial femoral cutaneous nerve. Most labelled cells were of the large, lateroventral type, but there were always some labelled cells which were smaller and situated mediodorsally. Therefore, cells from both the lateroventral and mediodorsal populations project to skin and to muscle, although individual neurones project to only one target (see below).

A small amount of cell death occurs in the d.r.g.s between St. 36 and St. 38, when it is complete (brachial d.r.g.s: Hamburger *et al.* 1981; lumbosacral d.r.g.s: M. G. Honig, unpublished data). However, it proved difficult to obtain retrograde labelling of cell bodies beyond St. 36 because deeper tissues become anoxic after several hours in the bath. Further, the small amount of cell death occurring after St. 36–37 is restricted to the less mature mediodorsal population (Hamburger *et al.* 1981). Therefore, even if the remaining cell death were to remove all the mediodorsal cells projecting to one or both types of targets at St. 36, which is unlikely, the results would not be substantially changed. That is, lateroventral cells would still project both to skin and to muscle, and the cell bodies of afferents projecting to skin and to muscle would still be widely distributed in the d.r.g.s.

The distribution of afferents projecting to the femorotibialis and adductor muscles was examined. Both lateroventral and mediodorsal neurones projected to each muscle, as shown in Fig. 3*E* and *F*. The distributions of labelled cells were fairly widespread in all cases and did not show a greater degree of localization when rostral, middle or caudal thirds of the ganglia were considered separately. Despite some clustering of labelled cells, for instance in the medial part of d.r.g. 2 for femorotibialis afferents (Fig. 3*F*), such clusters were not found consistently in the same location. Widespread distributions of labelled cells were also seen upon casual examination of HRP fills of several other muscles (see Landmesser, 1978*a*) including those innervated by d.r.g.s 3–8, and thus seem to be a general feature of d.r.g. organization.

The choice of the sartorius, femorotibialis and adductor muscles allowed comparisons to be made for three distinct properties of the muscles. First, there was no correlation between cell body position in the ganglia and topographical relationships of muscles in the adult limb. Secondly, the distributions of cell bodies for the ventrally derived adductor as compared to the dorsally derived sartorius and femorotibialis (Romer, 1927) were similar. Finally, although the sartorius is a flexor whereas the femorotibialis and adductor are extensors (Landmesser, 1978*a* and unpublished observations), their cell bodies were not localized in distinct regions of the ganglia.

It is clear, then, that there is always sufficient overlap in the positions of cells projecting to different targets that the position of a cell cannot be used to predict its target.

The relatively low numbers of labelled mediodorsal cells observed may be explained in several ways. First, d.r.g.s 1 and 2 project to several other limb muscles (e.g. obturator, iliotibialis) and cutaneous nerves, as well as axial musculature and cutaneous nerves which were not injected. Secondly, the localization of sympathetic afferents as well as afferents to tendons, joints, etc. has not been determined. Thirdly, the scarcity of labelled cells in the mediodorsal region may be in part due to the greater difficulty in visualizing HRP labelling in smaller cells. However, in spite of the low numbers of labelled mediodorsal cells, since some mediodorsal cells projected along the cutaneous nerves and others projected to muscles, they cannot correspond to a single cell type.

Neurone clusters. Labelled afferent cell bodies were frequently found adjacent to each other. Cell division continues after the initial formation of the d.r.g. and little migration takes place within the d.r.g. itself at least after St. 34; that is, the general spatial distribution of neurones with selected birthdates is similar in 10 d (St. 36) and

TABLE 1. Clustering of cells within ganglia (% of total cells in clusters of each size)

	No. of labelled cells	Single cells	2 s	3 s	4 s	5 s	6 s	7 s	All size clusters	Predicted % of pairs for random distribution
St. 35 adductor										
d.r.g. 1	129	82·9	12·4	4·7	0	0	0	0	17·1	0·37
d.r.g. 2	237	71·7	19·4	8·9	0	0	0	0	28·3	1·2
d.r.g. 3	6	100·0	0	0	0	0	0	0	0	0·000008
d.r.g. 4	2	100·0	0	0	0	0	0	0	0	0·0000009
St. $30\frac{1}{2}$ adductor										
d.r.g. 1	2	0	100·0	0	0	0	0	0	100·0	0·000008
d.r.g. 2	47	68·1	12·8	19·1	0	0	0	0	31·9	0·0044
d.r.g. 3	12	83·3	16·7	0	0	0	0	0	16·7	0·00029
d.r.g. 4	3	100·0	0	0	0	0	0	0	0	0·000018
St. 36 lateral femoral cutaneous										
d.r.g. 1	385	65·5	22·9	5·5	2·1	2·6	1·6	0	34·5	3·3
d.r.g. 2	465	53·8	21·9	7·8	6·9	4·3	3·9	1·5	46·2	4·8
d.r.g. 3	20	80·0	0	0	0	0	0	0	20·0	0·00009
St. 29 lateral femoral cutaneous										
d.r.g. T7	28	50·0	35·7	0	14·3	0	0	0	50·0	0·0016
d.r.g. 1	178	49·4	32·6	15·2	0	2·8	0	0	50·6	0·063
d.r.g. 2	83	63·9	28·9	7·2	0	0	0	0	36·1	0·014

18 d embryos (M. McPheeters & L. M. Okun, personal communication) and even in embryos as young as St. 34 (M. G. Honig, unpublished data). It is therefore probable that cells adjacent to each other in the ganglion often will be the progeny of the same mother cell. To examine this possibility, the number and size of clusters of labelled neurones projecting along the lateral femoral cutaneous nerve and to the adductor muscle were counted (Table 1). At St. 35–36, after most cell death has occurred, 17–46 % of the labelled cells in individual ganglia were found in pairs and small groups. This high incidence of clusters is striking since only a few hundred cells were generally labelled in ganglia containing roughly 7000–10,000 neurones each (Levi-Montalcini & Hamburger, 1951; Mitolo *et al.* 1965; M. G. Honig, unpublished observations). Except for those ganglia containing fewer than ten labelled cells, the percentages of pairs counted were many times greater than those expected for a random distribution of labelled cells (see Methods). The occurrence of clusters containing three or more adjacent labelled cells also suggests that the distribution of labelled cells is not random. Since one or more cells in a cluster initially projecting to a given target might die during the period of cell death, more clustering would be expected at earlier stages. In fact, in the St. 29 and St. 30½ embryos examined, the percentages of labelled cells found in clusters were larger than at older stages while the predicted percentages were even lower.

Thus labelled cells are found adjacent to each other much more frequently than would be expected if labelled cells were randomly distributed in the d.r.g.

The development of the segmental projection pattern

The results described thus far indicate that there is no correlation between target site in the limb and position of the cell body within individual d.r.g.s. The widespread and overlapping distributions of cell bodies projecting to different targets mean that the 'identities' of individual neurones cannot readily be ascertained. Experiments were carried out to determine if a specific segmental pattern of projections could be used to identify sensory neurones. First, the segments that give rise to the innervation of each target were identified and their contributions were quantified. Secondly, the peripheral projections of afferents originating from each segment were determined.

Spinal nerve contributions to individual targets in mature (St. 36–38) embryos. The contributions of each of the eight spinal nerves to individual targets were electrophysiologically determined at St. 36 and St. 38, after all motoneuronal (Hamburger, 1975) and most sensory (Hamburger *et al.* 1981 and see above) cell death. The results from the two stages were similar ($P > 0{\cdot}1$) and will be considered together.

The relative segmental contributions to the two cutaneous nerves were distinctly different (Fig. 4). The large compound action potential responses to stimulation of LS1 and LS2 indicate that the lateral femoral cutaneous nerve receives most of its axons from d.r.g.s 1 and 2 and only a small contribution from d.r.g. 3. The innervation of the medial femoral cutaneous nerve arises primarily from d.r.g. 2 with smaller contributions from d.r.g.s 1 and 3 (Fig. 4*B*). This general pattern of cutaneous projections was found consistently when the results from thirteen St. 36–38 embryos were quantified (Table 2). There was, however, considerable variability between embryos. For example, although the average contribution from LS1 to the lateral femoral cutaneous nerve was greater than that from LS2 ($P \leqslant 0{\cdot}05$), this was not the

case in all embryos. Further, a small contribution from T7 was sometimes but not always present. Nevertheless, the contribution from LS1 to the lateral femoral cutaneous nerve was always many times greater than that from LS3. This was confirmed by HRP fills of the nerve in three St. 36–37 embryos (Table 2).

Responses to spinal nerve stimulation recorded from the obturator and sartorius muscle nerves indicated that axons projecting along the obturator nerve originate

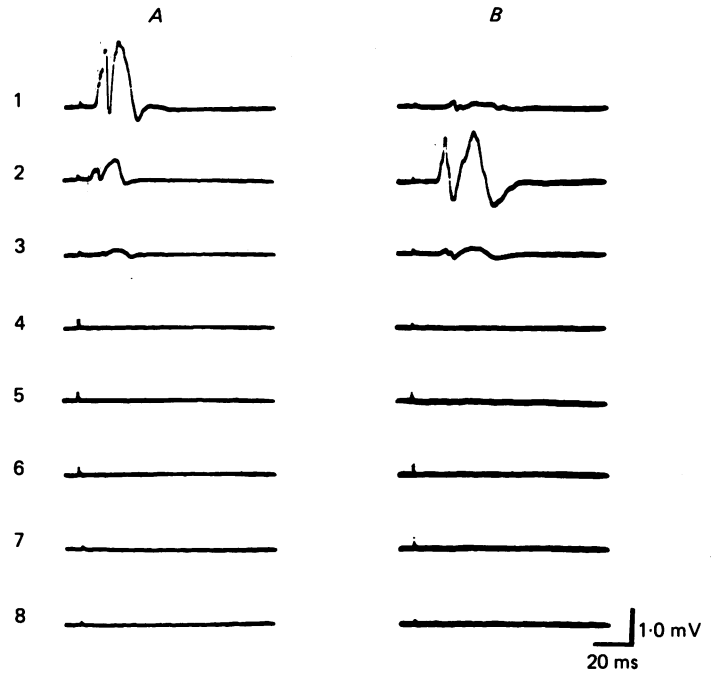

Fig. 4. Compound action potential recordings from the lateral femoral cutaneous nerve (*A*) and the medial femoral cutaneous nerve (*B*) elicited by supramaximal stimulation of lumbosacral spinal nerves 1–8 in a St. 36 embryo.

mostly from LS2 ($P \leqslant 0{\cdot}01$) with smaller contributions from LS1 and LS3 (Table 3*A*). Axons to the sartorius arise mostly from LS1 and LS2, as previously shown by Landmesser & Morris (1975). The average contribution from LS1 was larger than that from LS2 ($P \leqslant 0{\cdot}01$) although this was not true in all individual cases. Since afferent and efferent contributions could not be distinguished by this method, they were independently assessed using retrograde HRP labelling.

HRP injection of the sartorius muscle labelled sensory neurones mostly in d.r.g.s 1 and 2 and motoneurones in the corresponding cord segments (Fig. 5 and Table 3*B*). Minor contributions from the adjacent segments T7 and LS3 were sometimes found. The segmental contributions determined by counting labelled cell bodies were very similar to those calculated from muscle nerve recordings (Table 3). The roughly equal numbers of motoneurones and afferents projecting to the sartorius (e.g. see Fig. 5) suggest that motoneurones do not dominate the compound action potential response.

Most neurones projecting to the femorotibialis muscle are localized in segments 2 and 3, with a smaller contribution from segment 1 (Fig. 5 and Table 3*B*). The average

TABLE 2. Segmental contributions to cutaneous nerves

A. % contribution from each spinal nerve to compound action potential

	T7	LS1	LS2	LS3	LS4	*LS5-LS8
Lateral femoral cutaneous nerve						
St. 36–38 ($n = 13$)	2·1 ± 5·9	57·6 ± 20·5	36·9 ± 19·7	3·6 ± 4·8	0	0
St. 31–33 ($n = 7$)	2·6 ± 3·6	63·3 ± 16·1	32·9 ± 18·0	1·1 ± 0·6	0	0
St. 29–30 ($n = 7$)	8·4 ± 10·9	63·8 ± 22·7	24·3 ± 22·4	3·5 ± 6·1	0	0
Medial femoral cutaneous nerve						
St. 36–38 ($n = 13$)	0·3 ± 0·1	17·2 ± 15·9	60·2 ± 14·6	22·3 ± 16·8	0	0
St. 31–33 ($n = 8$)	0·1 ± 0·1	17·0 ± 16·6	58·3 ± 10·4	23·9 ± 14·6	0	0
St. 29–30 ($n = 8$)	0·5 ± 1·3	23·5 ± 10·4	63·8 ± 9·2	12·2 ± 7·8	0·4 ± 1·1	0

B. % of total HRP labelled cells found in each d.r.g.

	T7	LS1	LS2	LS3	LS4	†LS5-L88
Lateral femoral cutaneous nerve						
St. 36–37 ($n = 3$)	4·9 ± 7·5	48·5 ± 9·1	44·7 ± 15·4	1·9 ± 1·1	0	0
St. 29 ($n = 4$)	3·4 ± 4·2	62·8 ± 14·9	27·2 ± 10·8	6·4 ± 8·5	0·2 ± 0·3	0

* Spinal nerves LS5, 6, 7 and 8 were in each case stimulated separately.
† Segments LS5, 6, 7 and 8 were in each case examined separately.
Values are mean ± S.D. n = number of observations.

TABLE 3. Segmental contributions to muscles

A. % contribution from each spinal nerve to muscle nerve compound action potential

	T7	LS1	LS2	LS3	LS4	*LS5-LS8
Obturator nerve						
St. 36–38 ($n = 10$)	0·1 ± 0·3	10·5 ± 8·3	64·1 ± 16·5	25·4 ± 21·2	0	0
St. 31–33 ($n = 6$)	0 ± 0	6·8 ± 5·6	67·1 ± 16·1	26·1 ± 20·5	0	0
St. 29–30 ($n = 7$)	0 ± 0	20·8 ± 22·2	61·3 ± 22·6	18·0 ± 24·4	0	0
Sartorius nerve						
St. 36–38 ($n = 9$)						
†p. sart	1·0 ± 1·9	64·8 ± 23·1	33·0 ± 23·9	1·6 ± 3·9	0	0
a. sart	0·8 ± 2·2	63·6 ± 18·8	34·5 ± 19·9	1·2 ± 3·4	0	0
St. 31–33 ($n = 5$)	3·4 ± 6·0	62·9 ± 20·8	29·9 ± 24·7	3·7 ± 5·9	0	0
St. 29–30 ($n = 5$)	10·6 ± 18·5	72·5 ± 12·3	15·8 ± 11·1	1·1 ± 1·4	0	0

B. % of HRP labelled cells in each segment (St. 35–37)

	T7	LS1	LS2	LS3	LS4	‡LS5-LS8
Sartorius ($n = 5$)						
Afferents	3·3 ± 4·2	54·2 ± 15·2	39·6 ± 17·6	3·0 ± 3·5	0	0
Motoneurones	4·6 ± 5·0	69·0 ± 14·6	25·7 ± 13·7	0·6 ± 0·6	0	0
Femorotibialis ($n = 4$)						
Afferents	0·08 ± 0·1	7·9 ± 8·5	36·1 ± 6·5	55·8 ± 12·1	0·2 ± 0·3	0
Motoneurones	0·08 ± 0·15	10·6 ± 3·2	41·9 ± 11·1	47·4 ± 12·8	0·1 ± 0·3	0
Adductor ($n = 6$)						
Afferents	0·2 ± 0·4	9·3 ± 12·5	61·3 ± 6·7	24·2 ± 13·3	5·0 ± 4·8	0
Motoneurones	0·03 ± 0·08	29·2 ± 18·6	62·5 ± 16·6	8·0 ± 3·8	1·2 ± 1·6	0

* Spinal nerves LS5, 6, 7 and 8 were in each case stimulated separately.

† At St. 36–38 recordings were made from both branches, 'anterior' and 'posterior' to the sartorius muscle. The two branches did not differ in their spinal nerve contributions. At St. 29–33, recordings were made only from the posterior branch.

‡ Segments LS5, 6, 7 and 8 were in each case examined separately.

Values are mean ± S.D. n = number of observations.

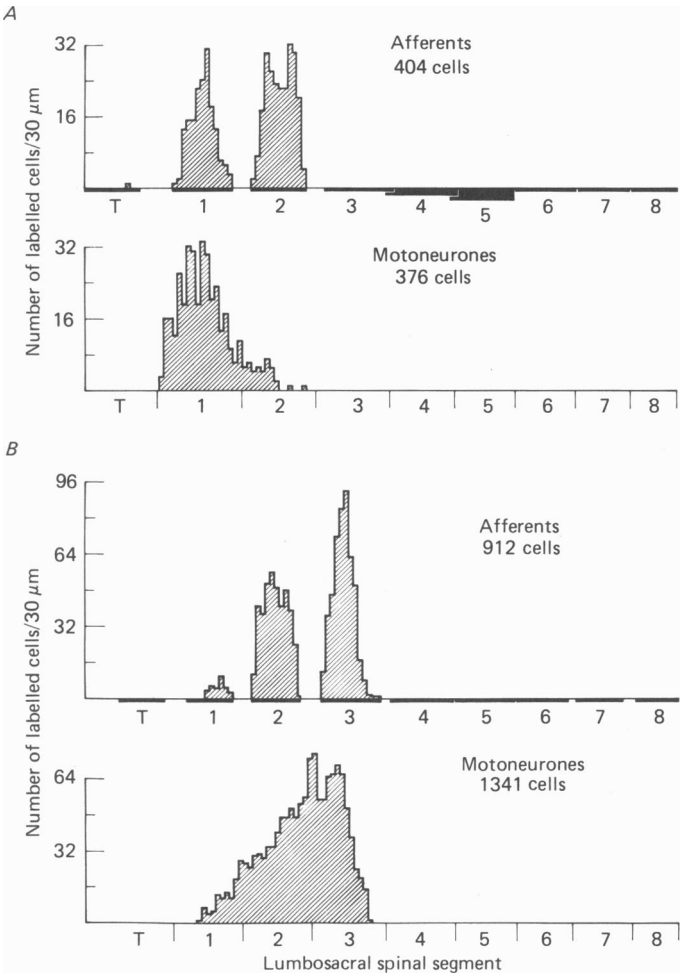

Fig. 5. Anterior–posterior positions of afferents and of motoneurones labelled by HRP injection of the sartorius (*A*) or femorotibialis (*B*) muscles at St. 36. Histograms show representative results from one embryo for each muscle. Black bars along the abscissa show the anterior–posterior extent of each d.r.g. Halfway between adjacent d.r.g.s was considered the end of one segment and the start of the next. T, the last thoracic segment, T7.

afferent contribution from LS3 is larger than that from LS2, but not significantly. The contribution from LS1 to the femorotibialis, not found in muscle nerve recordings (Landmesser & Morris, 1975), may represent leakage to the ambiens (see Landmesser, 1978*a*).

The adductor muscle receives most of its innervation from segment LS2 and additional contributions from the adjacent segments LS1 and LS3 (Fig. 6*A* and Table 3*B*). The presence of labelled cells in LS4 is probably the result of leakage to the adjacent ischioflexorius or underlying muscles, since stimulation of LS4 does not elicit a response in the obturator nerve which supplies the adductor (Table 3*A*).

For the sartorius, femorotibialis and adductor muscles it is clear (Figs. 5 and 6 and

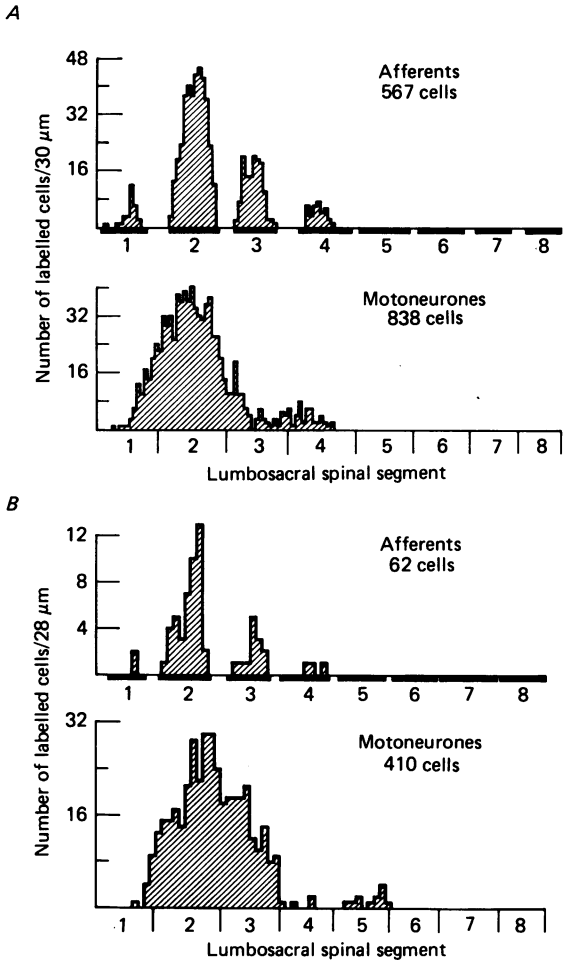

Fig. 6. Anterior–posterior positions of afferents and motoneurones labelled by HRP injection of the adductor muscle at St. 36 (*A*) and St. 30½ (*B*). These examples show the greatest amount of labelling in segments 4 and 5 that was ever found and which probably results from leakage to adjacent muscles (see text).

Table 3*B*) that afferents to a given muscle are localized in the same few segments as the motoneurone pool for that muscle. However, there is a small caudal shift of afferents with respect to motoneurones projecting to the same targets (see Table 3*B*).

Afferent projections to individual muscles are not significantly more widespread than the motoneurone projections. Although labelled d.r.g. cells were found in segments which did not have labelled motoneurones following six out of fifteen muscle injections, the additional contribution was always from a segment adjacent to the rest of the innervating segments and was quite small, generally less than 1 % of the total afferent supply to a muscle (fewer than six cells). It is possible that some of these cells might have been labelled as a result of slight HRP leakage.

A general pattern of projections for each spinal nerve also emerges from this data (Table 3*B*). For instance, LS1 provides the sartorius with a greater proportion (54·2 % afferents; 69·0 % motoneurones) of its total complement of axons than it does the

adductor (9·3 % afferents; 29·2 % motoneurones) or the femorotibialis (7·9 % afferents; 10·6 % motoneurones). When the sizes of each motoneurone and afferent pool are taken into account, it also becomes clear that LS1 sends more axons to the sartorius than to the adductor or femorotibialis and LS3 sends more axons to the femorotibialis than to the sartorius or adductor. However, for LS2 the situation is more variable and in some embryos this segment may supply the three muscles with similar numbers of axons.

Spinal nerve contributions to individual targets in St. 29–34 embryos. To determine if the early outgrowth of afferents into the limb is selective, projection patterns were assessed at St. 29–30, before most sensory cell death (Hamburger *et al.* 1981; data are for brachial ganglia) and at St. 31–33. The general pattern of cutaneous nerve projections at these stages was similar to that in mature embryos (Table 2). Nerve recordings showed the same relative contributions of each spinal nerve to each cutaneous nerve (e.g. for the lateral femoral cutaneous nerve LS1 > LS2 > LS3) described previously for St. 36–38 embryos. Furthermore, the contributions of each spinal nerve to each cutaneous nerve were generally similar at different stages. Analysis of HRP fills of the lateral femoral cutaneous nerve in four St. 29 embryos also gave results generally similar to those from older embryos (Table 2).

To examine the early outgrowth of afferents projecting to muscles, responses were recorded from the sartorius and obturator nerves at St. 29–30 and St. 31–33 (Table 3*A*). Although motoneurones undergo cell death from St. 29–35 (Hamburger, 1975), since they always project to the correct muscles (Landmesser & Morris, 1975; Landmesser, 1978*b*; Lance-Jones & Landmesser, 1981*a*), any difference in muscle nerve recordings between St. 29–33 and St. 36–38 should be attributable to inappropriate sensory projections. The contributions of the spinal nerves to the sartorius and to the obturator were in general similar to those found in older embryos when both the relative contributions of the various spinal nerves and the contribution of a given spinal nerve at different stages were compared.

Some small differences were present between different stages with respect to the average spinal nerve contributions to both cutaneous and muscle nerves. However, projections did not appear to be more widespread or diffuse in individual St. 29–30 embryos. Rather, it seemed that in some embryos the over-all innervation pattern was shifted either rostrally or caudally in comparison to 'normal'. Thus, the presence of a few HRP labelled cells in d.r.g. 4 in one St. 29 embryo (Table 2) was probably the result of a caudal shift of the entire projection rather than a 'developmental error'. Similarly, the larger average contribution of T7 and LS1 to the sartorius at St. 29–30 as compared to older stages (Table 3*A*) was probably due to a rostral shift of projections in three of the five embryos, which was also evidenced in the larger contribution of LS1 to the medial femoral cutaneous nerve (Table 2) and to the obturator nerve (Table 3*A*) in those embryos.

The distribution of afferents in the d.r.g.s projecting to the adductor and sartorius during the period of cell death was determined following HRP injections into these muscles. As shown in Fig. 6, the innervation of the adductor at St. 30½ is similar to that found in mature embryos. An appropriate segmental projection pattern was also found to the adductor in two additional embryos at St. 32 and 33½ and to the sartorius in five embryos ranging from St. 30½–34.

Thus early afferent innervation appears to be appropriate and cell death does not alter the segmental projection pattern in any substantial way. None of these studies, however, can rule out the possibility of inappropriate projections by some neurones

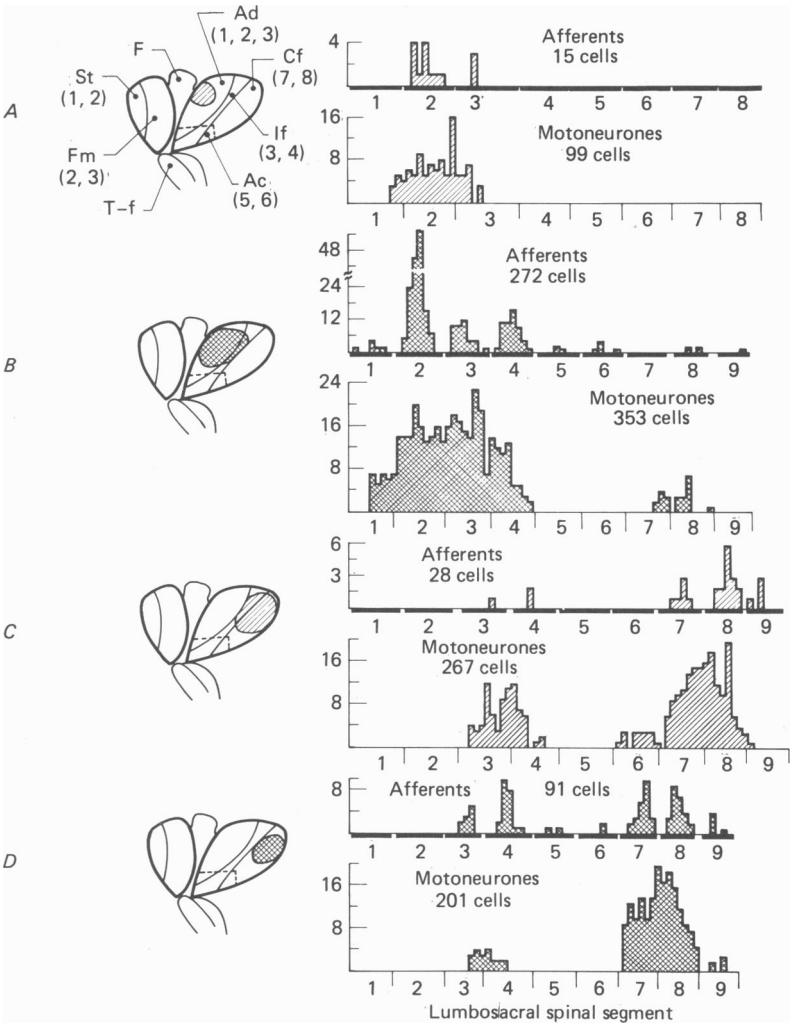

Fig. 7. Anterior–posterior positions of labelled afferents and motoneurones following partial injections of different parts of the ventral muscle mass of the thigh at St. 28. Diagrams show medial views of St. 28 limbs with approximate boundaries of prospective muscles delineated and injection sites outlined. Diagram at *A* shows lumbosacral segments making major contributions to mature muscles in parentheses. Ordinate shows number of labelled cells per 28 μm. The total number of labelled cells following each injection is also indicated.

St, sartorius; Fm, femorotibialis; Ad, adductor; If, ischioflexorius; Cf, caudilioflexorius; Ac, accessory; F, femur; T–f, tibia–fibula.

in appropriate ganglia. That is, for instance, some sartorius afferents in d.r.g.s 1 and 2 might initially innervate the adductor but they would not be distinguishable from adductor afferents in d.r.g.s 1 and 2.

Afferent projections to regions of the undivided muscle mass. Although afferents appear to grow down the appropriate nerves (at least from St. 29) and into the appropriate muscles from St. 30½ on, the possibility remains that prior to muscle cleavage (Romer,

1927; Landmesser, 1978*b*) there is widespread branching of axons in the undivided embryonic muscle masses. To examine this possibility a series of injections was made into different regions of the ventral muscle mass of the thigh in St. 28 embryos.

Injections into a small region of the uncleaved muscle mass labelled d.r.g. cells and motoneurones (see also Landmesser, 1978*b*) in the same segments as those labelled by similar injections of the muscles derived from that region (e.g. compare Fig. 7*A*

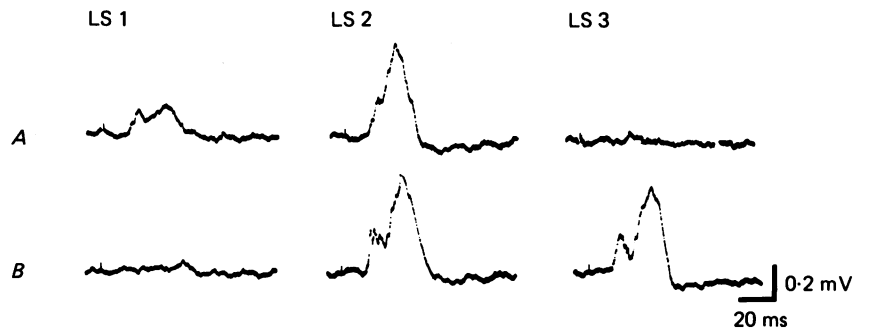

Fig. 8. Compound action potential recordings from lumbosacral spinal nerves elicited by supramaximal stimulation of the lateral femoral cutaneous nerve (*A*) and the medial femoral cutaneous nerve (*B*) in a St. 36 embryo.

with Fig. 6). Injections into the anterior part of the ventral muscle mass (which becomes the adductor) labelled d.r.g. cells in segments LS2 and 3 (Fig. 7*A*, *B*) whereas injections into the posterior part (which becomes the caudilioflexorius) primarily labelled cells in LS7 and 8 (Fig. 7*C*, *D*). Furthermore, as already shown for individual muscles after cleavage, the afferents labelled by each injection were located in the same segments as were the motoneurones. Thus, the majority of afferents as well as motoneurones (see also Landmesser, 1978*b*) seem to terminate in appropriate regions of the undivided muscle mass. Therefore, neurones must not only be able to grow down the appropriate nerve branches but also must ramify only within certain regions of the muscle mass even before there are any obvious physical borders.

Projections of individual ganglia in mature (St. 36–38) embryos. Projection patterns were also assessed by recording the compound action potentials from individual spinal nerves while successively stimulating the two cutaneous nerves. Fig. 8, typical of the results, shows that LS2 contributes roughly equivalently to both nerves. In contrast, many more neurones in d.r.g. 1 send their axons out the lateral femoral cutaneous nerve than out the medial femoral cutaneous nerve, while for d.r.g. 3 there are more axons projecting along the medial femoral cutaneous nerve than along the lateral femoral cutaneous nerve. This result was found in all St. 36–38 embryos examined (Table 4). In addition, in virtually all animals there is a shift in the relative contributions to each cutaneous nerve from one spinal nerve to the next. For example, the percent axonal contribution to the lateral femoral cutaneous nerve is greatest for LS1, intermediate for LS2 and smallest for LS3.

Early projections of individual ganglia. In order to examine outgrowth into the limb prior to St. 29, HRP was injected into individual ganglia and the presence of stained axons in the various peripheral nerves was assessed.

At early stages, stained axons extended into the limb as far as did the unlabelled axons, many of which arose from motoneurones. When individual peripheral nerve branches were first clearly distinguishable at St. 26½ (see also Lance-Jones & Landmesser, 1981*a*), both cutaneous and muscle nerves had formed. Since cutaneous nerve branches do not even at early stages contain any motoneurone axons (Lance-Jones & Landmesser, 1981*a* and personal communication), these observations suggest there is little, if any, delay between motoneurone and afferent outgrowth.

TABLE 4. Relative contribution of lateral femoral cutaneous nerve to LS1–3

A. % contribution to compound action potential*

	LS1	LS2	LS3
St. 36–38	82±10 (n = 10)	46±21 (n = 14)	18±14 (n = 7)
St. 31–33	93±9 (n = 6)	47±14 (n = 6)	4±7 (n = 5)

B. % of labelled axons following HRP injection of d.r.g.†

	LS1	LS2	LS3
St. 30–32	82±11 (n = 6)	30±13 (n = 3)	0±0 (n = 4)
St. 27–29	75±15 (n = 15)	31±9 (n = 6)	15±12 (n = 10)

* The sum total of the compound action potentials elicited by stimulation of the lateral femoral cutaneous nerve and of the medial femoral cutaneous nerve was assigned a value of 100%.

† The total number of labelled axons in the lateral femoral cutaneous nerve and the medial femoral cutaneous nerve was assigned a value of 100%.

Values are mean±S.D. n = number of observations.

This finding also implies that at least some sensory neurones are capable of following pathways in the limb independently of motoneurones. The lateral femoral cutaneous nerve reaches the skin by St. 27. The medial femoral cutaneous nerve, which first grows down the length of the thigh, enters the skin at around St. 27½.

This technique also provides information on the pathways axons take to reach their targets. The widespread distributions of sensory axons in the spinal nerves (Pl. 1*A*) suggest that they are already extensively intermingled with motoneurone axons. As spinal nerves LS1–3 converge in the crural plexus, labelled axons from each spinal nerve generally maintain their relative anterior–posterior positions (see also Lance-Jones & Landmesser, 1981*a*; Stirling & Summerbell, 1979; Ueyama, 1978). D.r.g. 1 axons course anteriorly in the crural plexus, d.r.g. 2 axons in the middle and d.r.g. 3 axons posteriorly (Pl. 1*B*). However, axons do not run strictly in parallel throughout their course. Spatial relationships between axons change and pathways of axons sometimes cross each other. For example, Pl. 1*C* shows labelled LS1 axons diverging, some maintaining an anterior position, while others (indicated by arrowheads) course posteriorly, crossing many unlabelled axons, to exit in the medial femoral cutaneous nerve.

HRP injections of d.r.g. 1 labelled many axons in the lateral femoral cutaneous nerve but only a few axons in the medial femoral cutaneous nerve. In contrast, injection of d.r.g. 3 resulted in more labelled axons in the medial femoral cutaneous nerve than in the lateral femoral cutaneous nerve. Estimates of the relative numbers of stained axons in the two cutaneous nerves showed that the relative contributions from each nerve were similar to those determined by recording from the spinal nerves

both in mature embryos and in a second series of embryos at St. 31–33 (see Table 4). Projections along muscle nerves were also similar to those found at later stages. Thus throughout the cell death period and even at St. 27–29, prior to cell death and when peripheral nerves are first distinguishable, the projections of ganglia are segmentally appropriate.

Therefore, afferents in particular ganglia projecting to both skin and muscle appear to grow down the appropriate peripheral nerves from the start. Sensory neurones choose a particular peripheral pathway before their central processes penetrate the grey matter of the spinal cord (Windle & Orr, 1934; M. G. Honig, unpublished results) and therefore presumably before they make any central connexions.

HRP was also injected into ganglia in St. 24–25 embryos, when axons have just begun to enter the limb bud. Axons arising from a given d.r.g. are then situated in generally appropriate regions of the crural plexus, for instance, d.r.g. 1 afferents occupy an anterior position. Therefore, there does not seem to be widespread testing of the environment by axons even at these early stages (see also Lance-Jones & Landmesser, 1981*a*).

Do afferents send branches to more than one target? The appropriate projection patterns found from St. 27 on suggest that large numbers of afferents do not send branches down more than one peripheral nerve early in development. This possibility was tested more directly in St. 29–32 embryos. The distribution of HRP labelled axons was examined after injection into one of the peripheral nerves. In seven embryos, labelled axons were restricted to the injected nerve. In two other embryos, a few (two to five) labelled axons were found in one nerve which had not been injected. In three out of six embryos examined, electrical stimulation of the medial femoral cutaneous nerve produced an axon reflex in the lateral femoral cutaneous nerve. In one other embryo stimulation of the sartorius muscle nerve elicited an axon reflex in the lateral femoral cutaneous nerve. In all cases the axon reflexes were very small in amplitude, ranging from 0·5 to 1·6% of the size of the total compound action potential for the peripheral nerve (see Methods). No detectable response was obtained in the lateral femoral cutaneous nerve to stimulation of the obturator (five cases), sartorius (three cases) or medial femoral cutaneous nerves (three cases). Such axon reflexes could reflect axon branches or arise from ephaptic interactions in the spinal nerves or plexus or from transmission across gap junctions between d.r.g. cell bodies (Pannese, Luciano, Iurato & Reale, 1977). Although the incidence of recording axon reflexes could be low because conduction can be blocked by placing d.r.g.s in suction electrodes, the similarity between the electrophysiological studies and the HRP results suggests that only a few afferents send branches down more than one peripheral nerve. Since some branched afferents may persist into adulthood (frog dorsal skin: Adrian, Cattell & Hoagland, 1931), there is no reason to suppose that the small degree of branching observed reflects an error in development.

Pathways of axons projecting to individual targets. The spatial relationships between axons projecting to a given target as they coursed through the spinal nerves and plexus were examined by tracing the pathways of labelled axons after retrograde HRP injection of individual peripheral nerves in St. 29–32 embryos. As shown in Fig. 9, in the proximal parts of the spinal nerves, labelled axons for any one target were widely distributed such that in spinal nerve 2, for instance, there was considerable

overlap in the positions of lateral femoral cutaneous, medial femoral cutaneous and sartorius axons. However, axons projecting out each nerve gradually segregated out together, with sartorius axons eventually taking up an anterior position in the plexus, lateral femoral cutaneous axons an anterior–lateral position and medial femoral cutaneous axons a posterior–medial position. These positions in the plexus

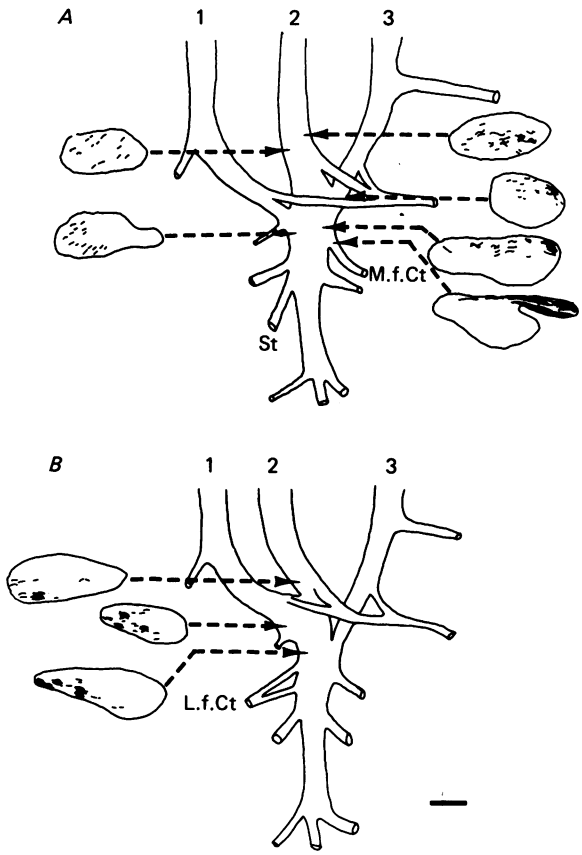

Fig. 9. The course of axons projecting out individual peripheral nerves shown by camera lucida tracings of nerve cross-sections at various levels from St. 30 embryos. *A*, the position of axons labelled by HRP injection of the sartorius muscle nerve (St) on the left (from Lance-Jones & Landmesser, 1981*a*) and of the medial femoral cutaneous nerve (M.f. Ct) on the right. *B*, the position of axons projecting to the lateral femoral cutaneous nerve (L.f. Ct). The most proximal nerve cross-section in each case is of spinal nerve 2 alone. The lateral surface of each nerve cross-section faces downward; anterior is to the left. Reconstructions of the plexus are schematic. Calibration bar is 50 μm for nerve cross-sections.

were characteristic of each nerve, although the level at which segregation of axons first became obvious varied somewhat between embryos.

As shown here for the sartorius and as found for other muscles (Lance-Jones & Landmesser, 1981*a*), the afferents and motoneurones projecting to a given muscle always sorted out together, forming a single group of axons.

DISCUSSION

The organization of the ganglion

These experiments show that neurones projecting along individual peripheral nerves are widely distributed within individual d.r.g.s and that the intraganglionic positions of afferent cell bodies are not related in any simple way to their target sites in the limb. Thus, contrary to the original assumptions of Hamburger & Levi-Montalcini (1949), both the large lateroventral and the small mediodorsal cells supply skin and muscle. Further, other recent experiments have indicated that several differences previously thought to exist between lateroventral and mediodorsal cells are also not real. For instance, both cell death (Carr & Simpson, 1978*a*, *b*; Hamburger *et al.* 1981) and responsiveness to nerve growth factor (Hamburger *et al.* 1981; Barde, Edgar & Thoenen, 1980) have now been observed within each population. Moreover, after mediodorsal cells differentiate and grow, the size differences between the two cell types are lost and large and small cells are found throughout the d.r.g.s (Hamburger & Levi-Montalcini, 1949). It therefore seems unlikely that there is any correlation between the two original cell types and modality, type of transmitter, (Hokfelt, Elde, Johansson, Luft, Nilsson & Arimura, 1976) or electrical properties (Yoshida & Matsuda, 1979). It is possible that the lateroventral and mediodorsal populations represent cells at different stages of development rather than two qualitatively different cell types.

The widespread distribution of neurones within each d.r.g. projecting to different targets suggests that there is also no correlation between target site and time of neuronal birth. There is a wave of birthdates in the d.r.g.s, with neurones at the lateroventral rim being born early, at St. 20–21, while neurones at the extreme mediodorsal pole are born much later, around St. 28–29 (M. McPheeters & L. M. Okun, personal communication). Thus the cells projecting to each target have a range of birthdates.

The establishment of the appropriate projection pattern

In spite of the lack of organization within individual ganglia, each d.r.g. sends axons down each of several peripheral nerves in a consistent and orderly pattern.

The basic segmental sensory projection pattern has been shown to be correct and precise from the earliest it can be examined, at St. 27, when distinct peripheral nerves are first clearly identifiable and before most cell death. Thus, the initial outgrowth of afferents is not random and the appropriate pattern of afferent projections is not established by the selective cell death of those afferents that had previously grown to inappropriate targets. This is also true for motoneurone innervation of the hind limb (*Xenopus*: Lamb, 1976; chick: Landmesser & Morris, 1975; Landmesser, 1978*b*; Lance-Jones & Landmesser, 1981*a*).

Several different mechanisms might explain how an appropriate pattern of *outgrowth* could be generated, as is discussed below.

Spatio-temporal mechanisms. A purely temporal hypothesis proposes that fibres entering the limb first occupy the most proximal pathways and those entering later occupy successively more distal and still available pathways (Jacobson, 1978). However, axons from the most rostral segments do not enter the limb before those

from more caudal segments, at least within the crural plexus. In addition, examination of the nerve pattern in young embryos indicates that axonal outgrowth to all targets starts at about the same time. Further, the finding that the cells projecting to each target have a wide range of birthdates suggests that axonal outgrowth along each peripheral nerve must occur over a prolonged period of time, concurrent with growth down other peripheral nerves. Therefore, this timed outgrowth hypothesis can be eliminated.

A second spatio-temporal hypothesis (Horder, 1978) requires that axons maintain constant topographical relationships with each other as they course through the spinal nerves, the plexus and into the limb. Nonspecific mechanical factors associated with limb morphogenesis then control the pattern of nerve branching, axons being channelled into nerves depending on their position in space and time. However, the results indicate that topographical relationships between axons are not constant in the spinal nerves and plexus. The pattern of innervation itself necessitates that the pathways of axons to different targets must cross extensively as has been seen when tracing the courses of afferents from single spinal nerves into the limb. Furthermore, the axons to a given target do not form individual fascicles within the spinal nerves which remain separate from other such groupings. Rather, and also in contrast to Horder's (1978) proposal, there is initial intermingling in the spinal nerves of motoneurones and afferents. This is followed by a gradual segregation of axons destined for the same target in a particular part of the plexus region.

Neuronal prespecification. Another hypothesis proposes that neurones possess distinct biochemical identities, or are 'pre-specified', and further that they are able to actively make use of environmental cues to grow to their appropriate targets, as seems to be the case for motoneurones in the chick hind limb (Landmesser, 1980; Lance-Jones & Landmesser, 1980). Sensory neurones could not be specified as a result of their ganglionic position (as motoneurones may be specified by their medial-lateral position in the lateral motor column: Landmesser, 1978*b*) or their time of origin (as suggested for retinal ganglion cells: Jacobson, 1968). However, specification could occur before the actual neurones under study are even born, as has been recently proposed for retinal ganglion cells (Gaze, Feldman, Cooke & Chung, 1979; Sharma & Hollyfield, 1980) and motoneurones (Lance-Jones & Landmesser, 1980, 1981*b*). If sensory neurones are specified before or during migration of the neural crest, the neurones could retain any acquired 'positional information' even if they subsequently alter their original spatial order. The resulting ganglia need not be topographically arranged, as is also true for other crest-derived ganglia (ciliary ganglia: Pilar, Landmesser & Burstein, 1980; superior cervical ganglia: Lichtman, Purves & Yip, 1979; cat d.r.g.s: Norcio & deSantis, 1976 but see Burton & McFarlane, 1973). Moreover, the clustering of cells projecting along common peripheral nerves observed here and in other crest-derived ganglia (Pilar *et al.* 1980; Norcio & deSantis, 1976) might result if sensory neurones were specified to innervate a particular target before their final mitosis, since adjacent neurones are likely to be the progeny of the same mother cell.

Two additional results are consistent with the possibility of an early specification. First, the gradual segregation of similar axons as they course from the spinal nerves to their individual targets might imply some sort of active recognition. This sorting

out could result from either interactions between axons or responses to some extrinsic factor(s), and seems to occur before any contact with the individual targets themselves. The alternative possibility that the segregation of axons seen at St. 29–32 results from the selective cell death of axons in other regions of the nerve which were not able to reach their target seems unlikely since no evidence for inappropriate innervation before St. 29 was found. Secondly, the similar segmental distributions of afferents and motoneurones innervating individual muscles is consistent with the hypothesis that the specification of afferents as well as motoneurones (Lance-Jones & Landmesser, 1980) is determined by their rostral-caudal positions at a time before neural crest migration. The slight caudal shift of afferents with respect to the corresponding motoneurone pool, found here and also reported for several muscles in the cat hind limb (Jefferson, 1954; Swett, Eldred & Buchwald, 1970; Hoffer, Stein & Gordon, 1979), could then be the result of later developmental events.

Guidance of afferents by motoneurones. All of the results presented here are, however, also consistent with several variations of a final mechanism in which sensory axons are guided to their target muscles by motoneurones. One possibility is that muscle afferents are specified to innervate particular muscles and are able to recognize and fasciculate with the appropriate motoneurone axons which guide them to the appropriate muscle. A second possibility is that some sensory neurones are specified to project to muscle but only in a general way and are guided by any motoneurone axon they happen by chance to contact. Finally, it is possible that afferents are not specified either as cutaneous or proprioceptive prior to outgrowth. Those afferents which grow down muscle nerves might do so either by fasciculating with motoneurone axons they contact by chance or by being passively channelled when motoneurone axons in their vicinity take a certain course. In all cases a specific segmental pattern of afferent contributions to individual muscles would result which would be similar to and determined by the motoneurone pattern.

The sensory neurones that grow down cutaneous nerves might similarly be specified to innervate skin and actively follow certain pathways. Alternatively, cutaneous afferents might simply be unable to interact with motoneurone axons and consequently be excluded from muscle nerves. A final possibility is that those initially unspecified afferents which do not contact motoneurone axons by chance might then become cutaneous. Even in this case, the segmental pattern of cutaneous projections could be specific. Sensory axons might be channelled first into particular areas of the plexus by mechanical factors perhaps associated in part with motoneurone outgrowth and subsequently into particular cutaneous nerves according to their position in the plexus. It is clear nevertheless that somehow axons must be able to choose between cutaneous and motor pathways and that motoneurones are always excluded from cutaneous nerves. Further, these experiments have shown that such exclusion could not be explained simply by temporal (Weiss, 1934) or spatial factors (Horder, 1978).

With this kind of mechanism there need not be a topographical arrangement of afferents in the d.r.g. since sensory neurones would not necessarily be specified prior to outgrowth. Moreover, small clusters of neurones might often project along the same peripheral nerve if the axons of adjacent neurones tend to contact each other and/or exit the d.r.g. in similar positions.

The questions of whether sensory neurones are unspecified even as cutaneous or

proprioceptive prior to outgrowth and whether interactions with motoneurone axons are necessary for generating a specific afferent projection pattern cannot be answered by any available evidence. These possibilities are now being tested by examining the outgrowth of sensory neurones in the absence of motoneurones.

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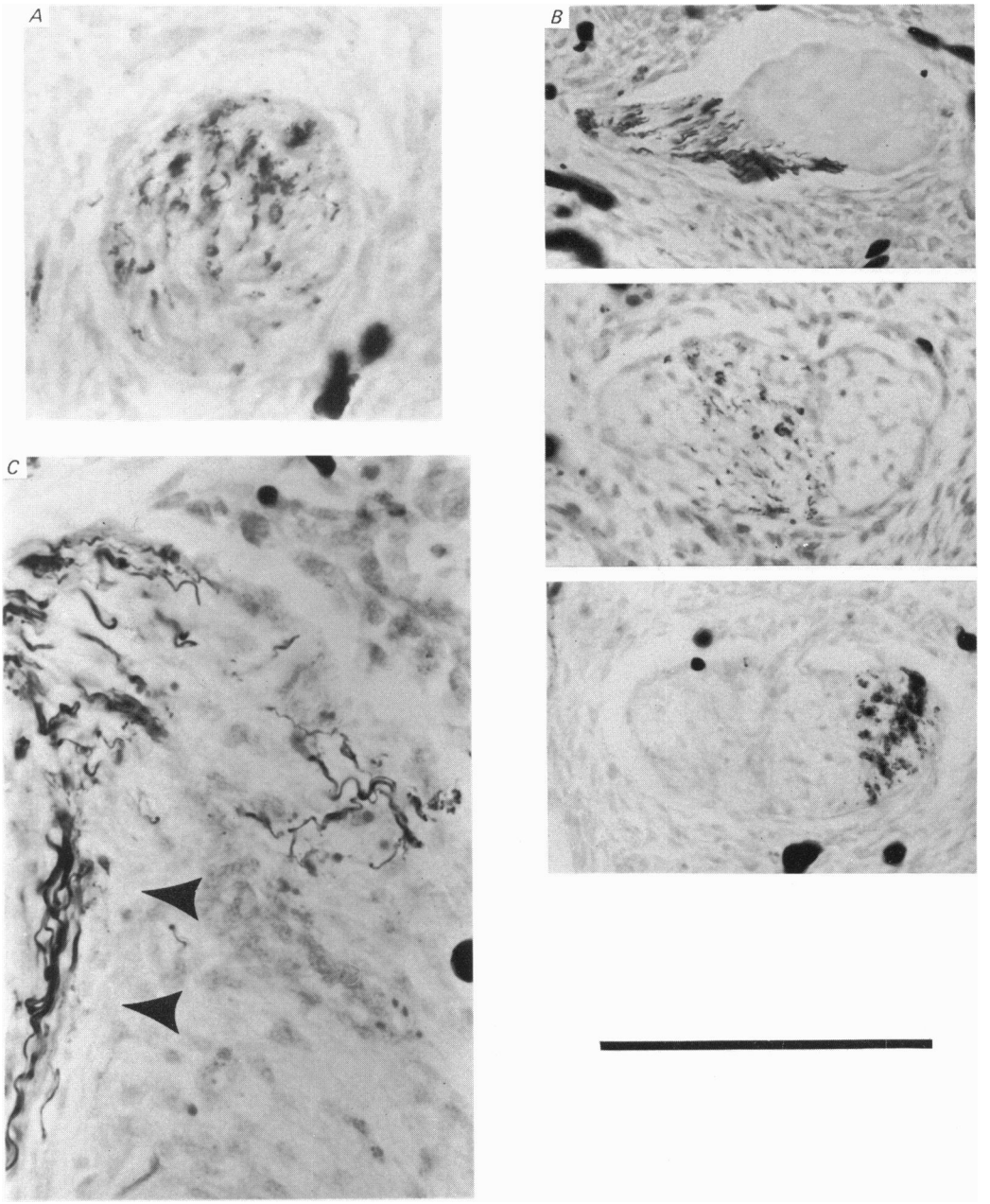
EXPLANATION OF PLATE

Photomicrographs of nerve cross-sections showing the position of axons labelled by injection of d.r.g. 1, 2 or 3 as indicated below. In *A*, axons labelled by HRP injection of d.r.g. 3 are spread throughout spinal nerve 3 and are intermingled with unlabelled axons. In *B*, at a level just distal to the emergence of the obturator nerve, axons labelled by HRP injection of d.r.g. 1 are localized in the anterior part of the crural plexus (top), d.r.g. 2 axons are situated in the middle part of the plexus (centre), and d.r.g. 3 axons in the posterior part (bottom). In *C*, axons labelled by HRP injection of d.r.g. 1 can be seen coursing in different directions and crossing over unlabelled axons and occasionally each other. Arrowheads indicate axons that are coursing posteriorly to exit in the medial femoral cutaneous nerve. Calibration bar, 80 μm for *A*, 100 μm for *B*, 65 μm for *C*.


A
B
C