

The precision of pathway selection by developing peripheral axons in the axolotl

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SUMMARY

At the time of hindlimb development in the axolotl there is a well-established but still developing trunk innervation. The trunk innervation is primarily composed of the segmental nerves, each of which consists of a dorsal and a ventral ramus and its branches. At a few segmental levels in the region of the hindlimb a large number of additional axons arise to innervate the limb. To reach the limb, they must grow via the ventral rather than the dorsal rami.

The precision with which this pathway is selected was determined by counting axons in the dorsal and ventral rami at limb segmental levels, prior to and during the period of maximum axon proliferation. If outgrowth is highly directed rather than random, then the ratio of ventral/dorsal ramus axons should increase significantly during the period when large numbers of additional axons are produced. In addition, since the dorsal trunk varies little in size between limb segments and immediately caudal 'non-limb' segments, the number of axons in the dorsal rami can be compared at the two levels. Mistaken projections should result in inordinately large axon numbers in dorsal rami at limb compared to non-limb levels.

The results show that there is approximately a tenfold increase in the ratio of ventral/dorsal ramus axons at the time of maximum outgrowth to the limb, thus the mode of distribution at the ventral–dorsal branch point is significantly altered in favour of growth toward the limb, and outgrowth appears to be highly directed rather than random. Moreover at this time there is no discernible increase in the number of dorsal ramus axons at limb levels while those at non-limb levels increase fourfold. The apparent growth of all axons into the ventral ramus suggests the presence of a strong, non-specific attraction.

INTRODUCTION

An increasingly conspicuous feature of nervous system development is the extension of developing axons into structures which they do not innervate in the adult (e.g. see Lamb, 1976; Land & Lund, 1979; McGrath & Bennett, 1979; Bunt & Lund, 1981; Frost, 1984). Moreover, many of these projections have been shown to form functional synapses before regressive processes (e.g. collateral withdrawal or cell death; Cowan, Fawcett, O'Leary & Stanfield, 1984; Purves & Lichtman, 1980) effect their removal.

Clarke (1981), has suggested that projection errors can be regarded as either 'mistakes' or 'imprecisions'. *Imprecisions* arise from a straying of axons or collaterals into regions which are in the near vicinity of the target area; these are potentially retrievable and therefore may not necessarily lead to death of the neurone. *Mistakes* encompass those projections which are grossly inappropriate

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when compared to the adult anatomy. The potential for these occurring seems greatest at critical points in the earlier stages of projection (for example at the optic chiasm) where the possibility of correction is remote.

If mistaken projections are devoid of *any* functional significance then they are particularly interesting in relation to the nature and efficiency of axon guidance mechanisms. Their existence suggests that the deployment of axons at branch points may be a random process rather than one which is highly controlled. While distribution of axon outgrowth through the optic chiasm is suggestive of random outgrowth of *single* axons (Land & Lund, 1979; Bunt & Lund, 1981; Jeffery, Gowrey & Kuypers, 1981), another strategy for pathway selection at branch points is suggested by the observation in the frog that collaterals form at plexus branch points and that many of these (probably inappropriate) collaterals are later withdrawn (Prestige & Wilson, 1980). This also appears to be the case in the axolotl (Freeman & Davey, 1984).

To further examine the various directional responses of axons at nerve branch points, an earlier phase of outgrowth to the axolotl limb was considered, where there would seem to be the potential for gross misprojections (such as those at the optic chiasm). Within each hemisegment, axons from the spinal cord and dorsal root ganglion combine to form the segmental nerve which consists of two major branches: the dorsal and ventral rami (Figs 1, 2); these innervate the portions of

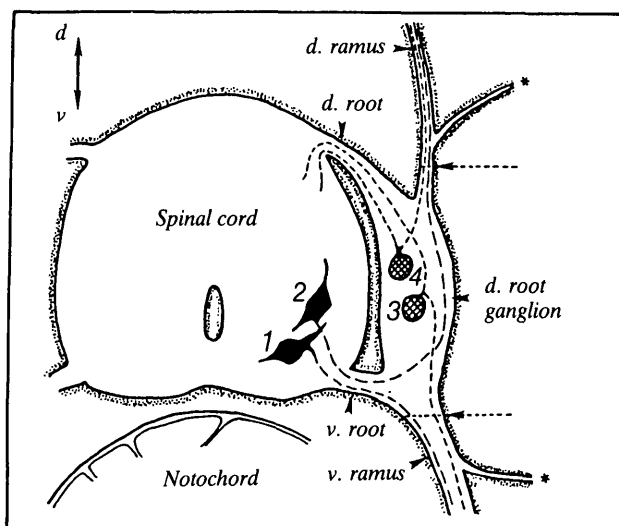


Fig. 1. A schematic transverse drawing of the nervous outflow from a single hemisegment showing the way in which sensory and motor axons contribute to each ramus. The motor cells in the ventral spinal cord (solid cell bodies and long-dashed axons) send single axons into the ventral (*v. ramus*) or dorsal (*d. ramus*) ramus (cells 1 and 2 respectively). The sensory cells (hatched cell bodies and short-dashed axons) send axons to the dorsal root (*d. root*) and to either the ventral or dorsal ramus (cells 3 and 4 respectively). The rami send extensive branches to the adjacent myotomes and the skin (*). The level at which axons were counted in the dorsal and ventral rami and indicated by the horizontal dashed lines.

the trunk lying dorsal and ventral to the spinal cord respectively (Francis, 1934). This trunk innervation is well established when the hindlimbs arise relatively late in development (Schreckenberg & Jacobson, 1975). If the large number of additional axons, which are generated at nearby segmental levels, are to follow the existing segmental nerves to reach the limb region, then they can only do so via the ventral rather than dorsal rami. It is this critical point in the extension of new outgrowth, where dorsal and ventral paths diverge, that is of particular interest in this study.

The description of the normal development of the segmental outflow to the trunk and hindlimb, and the detection of mistaken projections was undertaken in

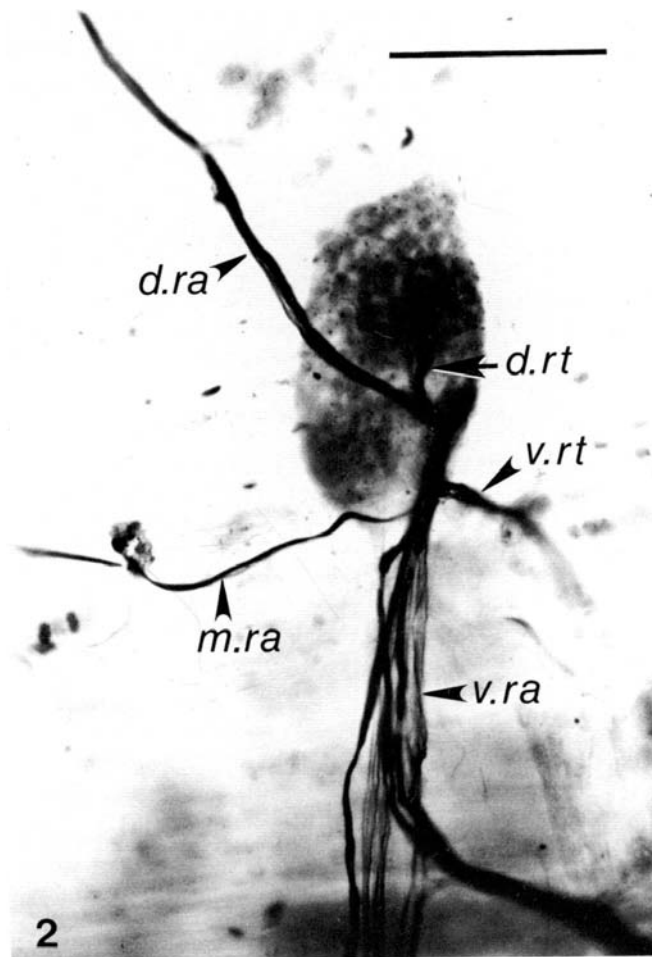


Fig. 2. A view of the medial surface of a whole-mounted dorsal root ganglion shows the dorsal ramus (*d.ra*) and ventral ramus (*v.ra*) of the segmental nerve. Very small medial rami (*m.ra*) are sometimes seen but usually only contain a few fibres. The overlying spinal cord has been removed and the severed dorsal and ventral roots (*d.rt*, *v.rt*) can be seen. The tissue was prepared using a Bodian silver stain according to the method of Lewis, Chevallier, Kieney & Wolpert (1981). Scale bar equals 100 μm .

the following way. Two regions of the axolotl trunk were considered: namely, those segments known to contribute to both trunk and limb innervation ('limb' segments) and those caudal to these which contribute to trunk innervation alone ('non-limb' segments). The ventral rami of segmental nerves 16 and 17 are known to be the major contributors to limb innervation, while smaller, variable contributions are made by levels 15 and 18 (Cass & Mark, 1975). In determining whether random outgrowth by axons or collaterals is a significant process at the point of dorsal-ventral divergence, the ratio of ventral/dorsal ramus axon numbers at limb levels was determined during the stages of peak outgrowth to the limbs. This was compared to the ratio at limb levels prior to axon proliferation as well as to the ventral/dorsal ratio at non-limb levels throughout development. Indeterminate distribution of the extra outgrowth arising at limb levels might be expected to result in ventral/dorsal ratios similar to those existing before significant outgrowth occurred, or to those at non-limb levels. Conversely, highly directed outgrowth would show a considerable bias in distribution in favour of the ventral ramus.

METHODS

Animals and staging

Wild-type axolotl (*Ambystoma mexicanum*) larvae from 14 to 30 mm body length were used. Seven animals were examined in detail to represent the period from the first appearance of hindlimb mesenchyme to when the limb has acquired two distinct digits. This latter stage is prior to the onset of hindlimb movement.

Animal length was used as the staging criterion since detailed staging only exists for embryonic animals (Schreckenbergs & Jacobson, 1975). Although limb morphology would be a desirable staging criterion, there are no distinctive limb features up to about the 21 mm stage. Our experience indicates that animal age is not a reliable indicator of larval development, since the rate of limb and length development is highly dependent on factors such as crowding, food availability and temperature. Adverse levels of any of these environmental conditions can slow development markedly. However, animals raised under adverse conditions when compared to those raised under favourable conditions, exhibit very similar states of limb development at the same body length; this is at least the case for stages included in this study.

Electron microscopy

Animals were anaesthetized in 0.03% w/v ethyl m-aminobenzoate (MS222; Sigma) in Holtfreter's solution (Hamburger, 1960) and a portion of whole trunk in the hindlimb region was dissected free.

The tissue was immersed (24 h, 4°C) in fixative based on that of Kalt & Tandler (1971), consisting of 2% w/v glutaraldehyde, 1% w/v paraformaldehyde, 0.5% v/v acrolein, 0.5 v/v dimethyl sulphoxide and 1 mM-CaCl₂ in 0.07 M-cacodylate buffer (pH 7.2). It was then washed (0.08 M-cacodylate buffer, pH 7.2, 4°C, 1 mM-CaCl₂) and postfixed in osmium (1% w/v OsO₄ in 0.07 M-cacodylate buffer, pH 7.2, 4°C) for 12–24 h. Tertiary fixation and staining was carried out in uranyl acetate (1% w/v in 0.05 M-maleic acid buffer, pH 5.4, room temperature). The tissue was dehydrated in alcohol and embedded in Spurr's (1969) resin using flat moulds. The block was viewed using bright-field and incident light microscopy with intense illumination and a *camera-lucida* drawing was made of a lateral view of the trunk tissue. Features such as the myotomal boundaries, the gut, the spinal cord and the limb were recorded for later identification of segmental levels in sectioned material.

The block was aligned so that thick (1 µm) horizontal sections through the dorsal myotomal muscle were cut in a plane parallel to the spinal cord. At the level of the dorsal extremities of the

dorsal root ganglia (Figs 1, 3A) thin sections displaying gold interference colours were cut to provide axon counts of dorsal rami. Sections were expanded under chloroform vapour and stained with lead and uranium salts following incubation in hydrogen peroxide (Pfeiffer, 1982). Serial $1\text{ }\mu\text{m}$ sections were then cut until the ventral extremities of the dorsal root ganglia were reached (Figs 1, 3B), i.e. where sensory and motor nerves have combined to form the ventral ramus. Thin sections were again taken to provide axon counts of the ventral rami at all segmental levels. Large scale *camera-lucida* drawings were made from the thick sections immediately adjacent to the thin sections and these were used to subsequently locate and identify nerve bundles using the electron microscope.

Morphometry

Rami were photographed at magnifications of 4000 or 6000 then photographically enlarged a further three times. Micrographs were combined into montages and identified by a code, so that all axon counts were performed blind. The number of axons in each ramus was determined with the aid of a digital computer and a digitizing tablet (Talos Simple One). Montages of whole

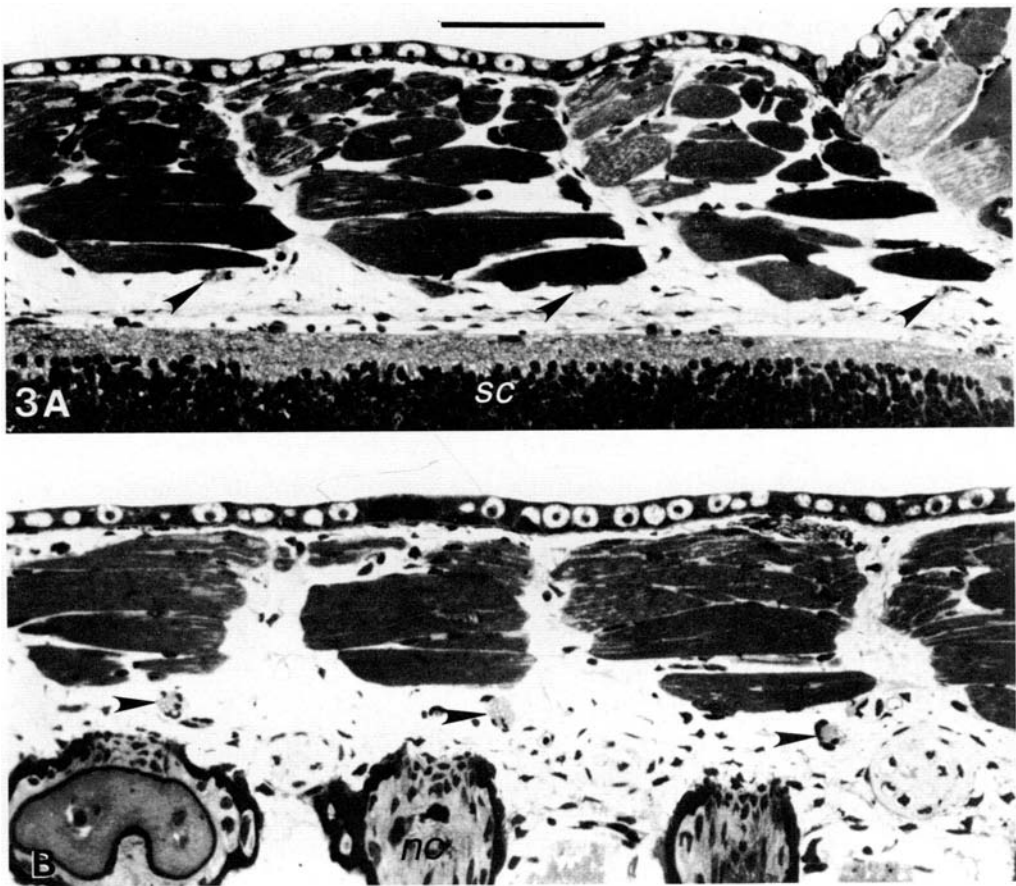


Fig. 3. Light micrographs of horizontal sections of a 26mm animal showing the location of dorsal (A) and ventral (B) rami (arrows) at the level at which counts were made. These levels correspond to the upper and lower dashed lines respectively in Fig. 1. Approximately three body segments are represented in each micrograph (sc, spinal cord; nc, notochord). Scale bar equals $200\text{ }\mu\text{m}$.

nerves were placed over the tablet and the minor diameter of each axon profile was traced using the digitizer stylus. The number of axons and the average axon diameter were computed for each ramus.

RESULTS

Axon counts of dorsal and ventral rami at hindlimb segmental levels and segments caudal to these are shown in Fig. 4. In one animal, counts were also made in segments rostral to the hindlimb region and these are shown in Fig. 5. While overall there is an increase in the number of axons as development proceeds, the rate of increase is not uniform across all segments. In particular, there is an accelerated growth in axons numbers in the ventral rami at segments 16 and 17. This coincides spatially and temporally with the development of the limb. A more subtle effect is the emergence at limb levels of a relative depression in dorsal ramus counts. This is best illustrated by the dorsal ramus counts shown in Fig. 5.

To further characterize these and other phenomena, axon counts of individual rami were pooled and averaged within four regions of the animal: namely dorsal and ventral rami at both 'limb' and 'non-limb' levels. Segments 16 and 17 were used to represent 'limb' levels and segments 20 and 21, 'non-limb' levels. In some animals both left and right sides were counted. Thus up to four individual counts, made up of bilateral counts at two segmental levels, contributed to the average value. Wherever axon counts of both rami of an individual segmental nerve were available, the ventral/dorsal ratio was calculated. For this purpose, segments caudal to segment 21 were also considered to be 'non-limb'. The following sections deal with axon count and ratio data plotted as a function of animal length.

The development of dorsal and ventral rami at non-limb levels

The development of trunk innervation in the absence of limb influences can be represented by axon counts of the dorsal and ventral rami at non-limb levels. These are shown in Fig. 6A. Although axons numbers generally increase steadily during development, the rate of growth of the dorsal rami appears to be relatively slow between the 15 and 21 mm stages. This is reflected in the ventral/dorsal ratio data (Fig. 6B). While being about 2:1 in the 14 mm animal, it quickly increases to about 4:1 before the 2:1 ratio is restored by 21 mm.

The development of dorsal and ventral rami at limb levels

The sharp increase in ventral ramus axon numbers between the 15 and 26 mm stages (Fig. 7A) corresponds to the period of limb development from the first appearance of mesenchyme in the trunk wall, through to the appearance of the paddle-shaped limb bud (see panel insets, Fig. 4). While the distribution of axons to the dorsal and ventral rami (Fig. 7B) is similar to that at non-limb levels at the earliest stage studied (14 mm; ratio about 4:1), the increased ratio beyond this point reflects the highly favoured outgrowth of axons into the ventral ramus. Moreover, the counts of axons in the dorsal ramus suggest that it is almost totally

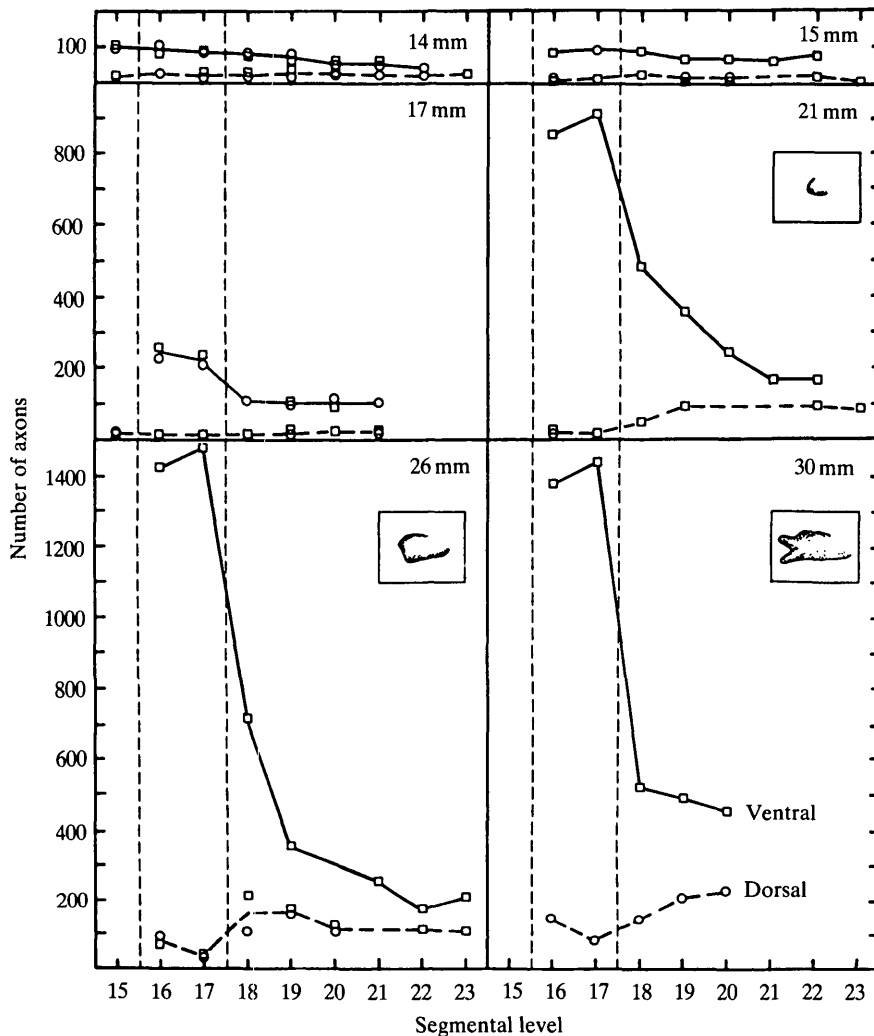


Fig. 4. Axon counts of ventral rami (solid lines) and dorsal rami (broken lines) were made at various stages of limb development. Each panel contains counts from a single animal. The stage of development is indicated by the animal length, and for older animals, a diagram representing the limb shape. The major contributions to limb innervation are made from segments 16 and 17. This segmental range falls between the vertical dashed lines. Smaller, variable contributions arise from segments 15 and 18, while segments 19 and onward appear to make insignificant or nil contributions. The curves are drawn through the mean of counts taken from the right (○) and/or left (□) side of the animal. The first significant increases in ventral ramus axon counts can be seen in the 17 mm animal at segmental levels 16 and 17. Subsequently, the rate of growth in axon numbers at these levels increases dramatically compared to non-limb levels. The large number of additional axons which are generated do not appear to project incorrectly down the dorsal ramus since the number of axons at these levels does increase significantly until the rate of outgrowth has declined. This is in contrast to non-limb levels where a significant amount of new outgrowth extends along the dorsal ramus. These results are summarized in Figs 6, 7 and 8.

avoided by outgrowing axons until the 26 mm stage. Certainly at the 21 mm stage, when the rate of increase in the number of ventral ramus axon numbers appears to be at its greatest, there is no discernible increase in the number of dorsal ramus axons, over that found at the 14 mm stage (Fig. 7A).

The comparison of outgrowth at limb and non-limb levels

If outgrowth at limb and non-limb levels is compared both before and during the period of axon proliferation at limb levels then some interesting results emerge which augment those described above. The validity of this comparison is argued in the Discussion.

Firstly, the difference in the number of ventral ramus axons between limb and non-limb levels (Fig. 8A), provides an estimate of the way in which outgrowth at limb levels is apportioned between trunk and limb targets. More significant, however, is the comparison between dorsal rami which suggests that the observed lack of outgrowth at limb levels is not a reflection of the innervation requirements

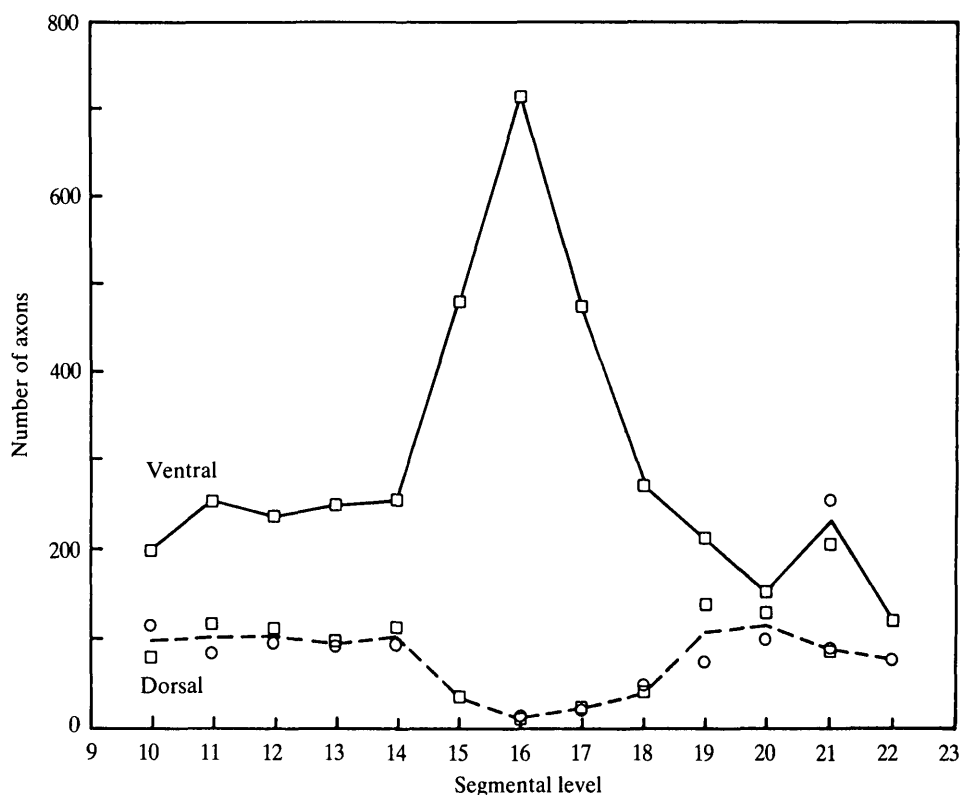


Fig. 5. Axon counts from the right (○) and/or left (□) sides of a single 20.5 mm animal include segments rostral as well as caudal to the limb levels. This shows that the depression in dorsal ramus numbers coincides precisely with the elevated ventral ramus counts at limb segmental levels. Note that the depression is a graded phenomenon and appears to be greatest at the segmental level where there is maximum ventral ramus outgrowth to the limb.

of the dorsal trunk since at non-limb levels, axon numbers steadily increase (Fig. 8B). Thus the dorsal trunk at limb levels *appears* to be deprived of new outgrowth, at least until the 26 mm stage. The relative depression is both spatially and temporally coincident with the emergence of peaks in ventral ramus outgrowth (Figs 4, 5). The appearance of the dorsal rami at limb compared to non-limb levels are significantly different and these are contrasted in electron micrographs (Fig. 9). The differences are particularly well represented in axon diameter data.

Changes in axon diameter during development

In addition to axon counts, the average diameter of axons in the various regions considered, was used to indicate developmental trends. Average diameter statistics are subject to two major developmental influences which are seen in plots of average diameter as a function of developmental stage. Maturation of existing outgrowth will tend to produce a positive slope, whereas new outgrowth added to established nerves gives rise to a negative slope because new outgrowth consists of small diameter axons. These two influences interact to produce a final slope which reflects the relative weight of each influence during development. In this study, measurements of average axon diameter reveal a wide spatial and temporal variation (Fig. 10). In the 14 mm animal, the average diameter of axons in each region is similar, however, these values quickly diverge as development proceeds. There is an early pronounced increase in the average diameter of dorsal rami axons at limb segmental levels. This increase coincides with the period during which the dorsal rami appear to be 'deprived' of new outgrowth. Conversely, the average diameter of axons in the ventral rami at limb levels decreases significantly during the period when there is extensive new outgrowth.

DISCUSSION

Are outgrowing axons at limb levels distributed randomly?

The results suggest that random outgrowth (followed by the death of incorrect projections; Summerbell & Stirling, 1982) is *not* the process used to achieve the appropriate final distribution of axons since the number of dorsal ramus axons at limb levels remains essentially constant during the period of maximum axon outgrowth (about 21 mm); this is when the highest incidence of errors would be expected to occur. Another perspective on this is gained by examining the ratio of ventral/dorsal ramus axon numbers which increases to about 50:1 at the time of peak outgrowth (Fig. 7B). This represents a significant deviation from the distribution which persists at non-limb levels (maximum ventral/dorsal ratio of 4:1; Fig. 6B) or that which existed at limb levels before extensive outgrowth began (also about 4:1; Fig. 7B, 14 mm body length). Thus the mode of distribution is significantly altered and appears to be anything but random.

There is the possibility that mistaken projections may have arisen at stages intermediate to those studied or that they may occur but withdraw before reaching the dorsal ramus sampling point. In the first case, such short-lived errors would be

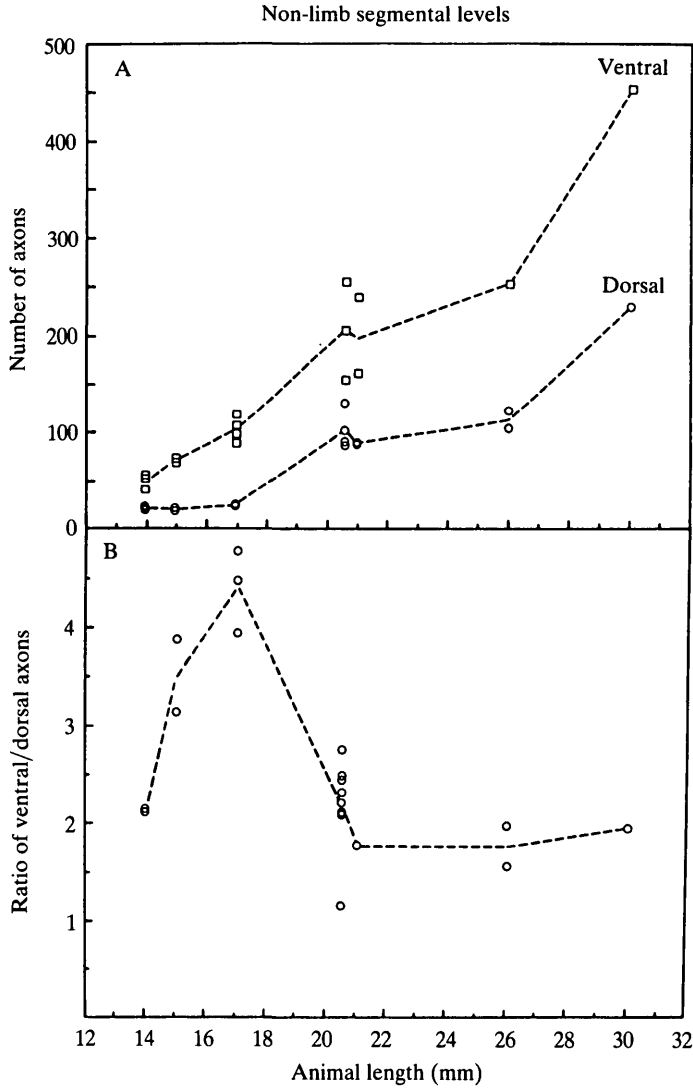


Fig. 6. (A) The relative contributions of dorsal and ventral rami to trunk innervation as the animal develops is shown by axon counts of dorsal (○) and ventral (□) rami at non-limb levels. Each datum represents a count of a whole ramus at segmental level 20 or 21. The lines are drawn through the mean of up to four points (from bilateral counts at two segmental levels). Note that there is no significant increase in dorsal ramus counts until the 21 mm stage. (B) The ratio of ventral/dorsal ramus axon numbers at non-limb levels in individual segmental nerves, are shown as a function of developmental stage (○). The counts were taken from segmental levels 20 and others caudal to this. The connecting lines are drawn through the means of the ratios. The ratio is close to 2:1 at 14 mm body length, but this undergoes a transient increase to about 4:1 before returning to 2:1 (by 21 mm) for the remainder of the period studied.

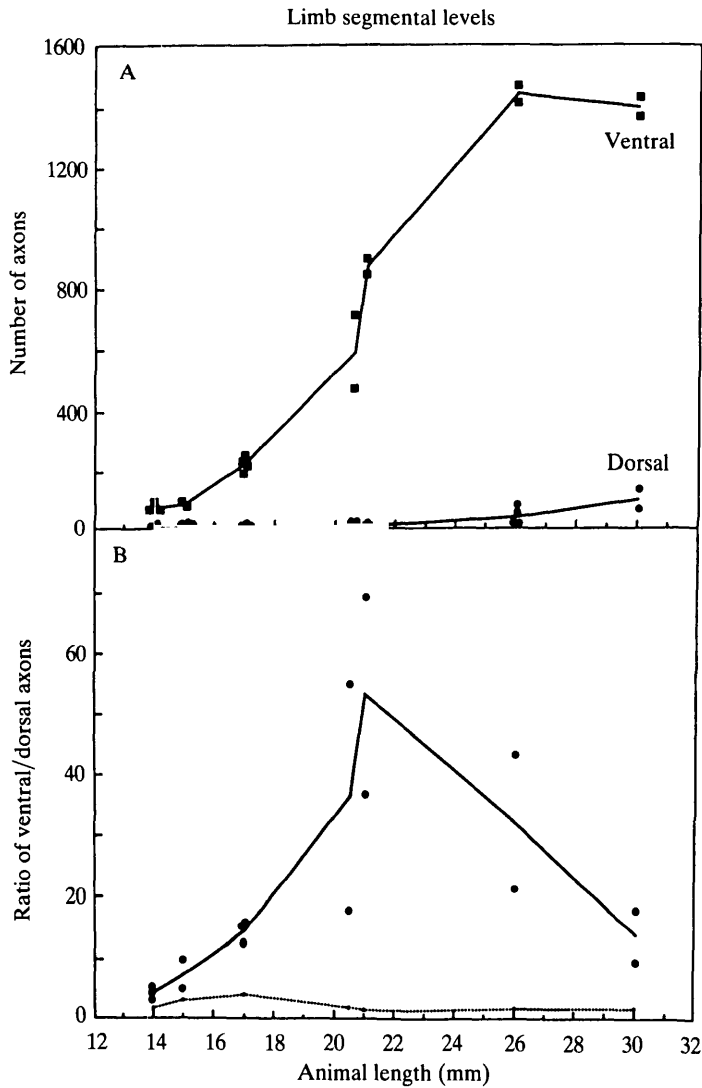


Fig. 7. (A) This figure illustrates the preferential growth of axons into the ventral (■), rather than dorsal (●) rami at limb levels. Most importantly, this preference persists during the period of the maximum rate of increase in axon numbers (21 mm stage). Each datum represents a count of a whole ramus at segmental level 20 or 21. The lines are drawn through the mean of up to four points (from bilateral counts at two segmental levels). (B) The ratio of ventral/dorsal ramus axon numbers at limb levels in individual segmental nerves, are shown as a function of developmental stage (●). Although the initial values are low and comparable to those at non-limb levels, the ratio increases by an order of magnitude by the 21 mm stage. This demonstrates a considerable change in the way in which axons are distributed between the two rami. The connecting lines are drawn through the means of the ratios. The ratios at non-limb levels are also plotted (----) for comparison.

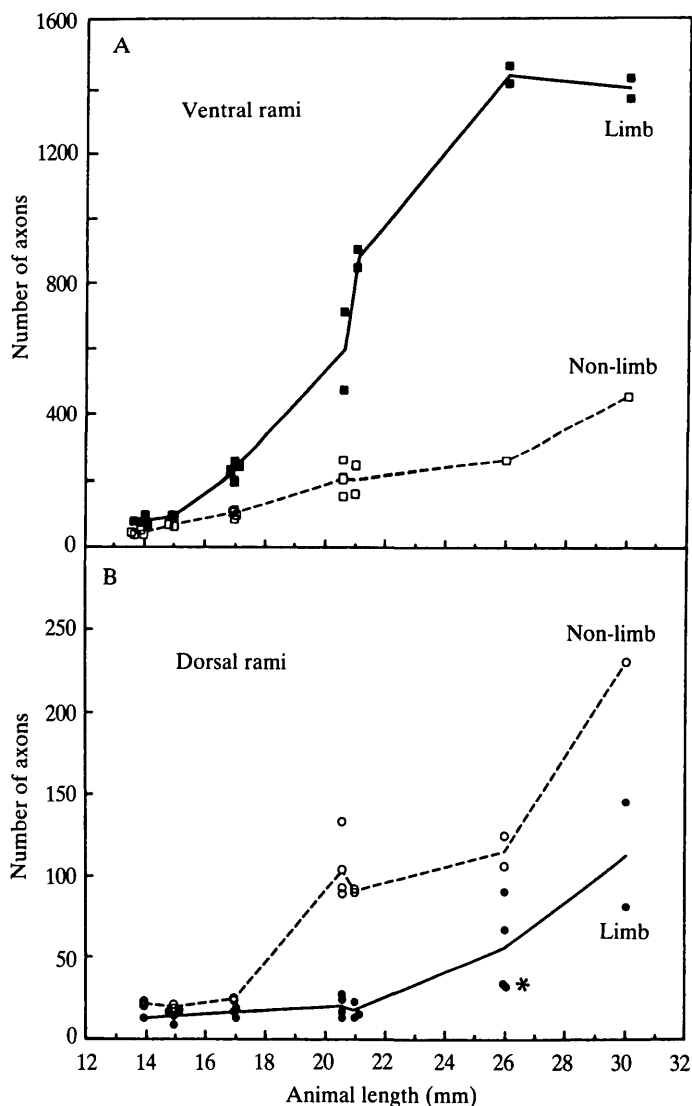


Fig. 8. (A) The number of additional axons which are generated at limb segmental levels can be assessed by comparing axon counts of ventral rami at limb (■) and non-limb (□) levels. Each datum represents a count of a whole ramus at segmental level 16 or 17 for limb levels, or, 20 and 21 for non-limb levels. The lines (solid, limb; dashed, non-limb) join the mean of up to four points (from bilateral counts at two segmental levels). The hind-limb bud is first visible as a small bulge at about 20 mm body length. (B) The absence of projection errors is shown by axon counts of dorsal rami at limb (●) and non-limb (○) levels as defined in (A). Projection errors should result in an inordinately high number of axons in dorsal rami at limb levels and this should be most obvious during the period of maximum outgrowth (about 21 mm). The results show instead that the counts at limb levels are significantly depressed in relation to non-limb levels. This is because dorsal ramus counts at limb levels do not increase significantly until the 26 mm stage and even this is mostly confined to level 16. The level 17 counts remain virtually unchanged (*). Meanwhile, counts at non-limb levels steadily increase.

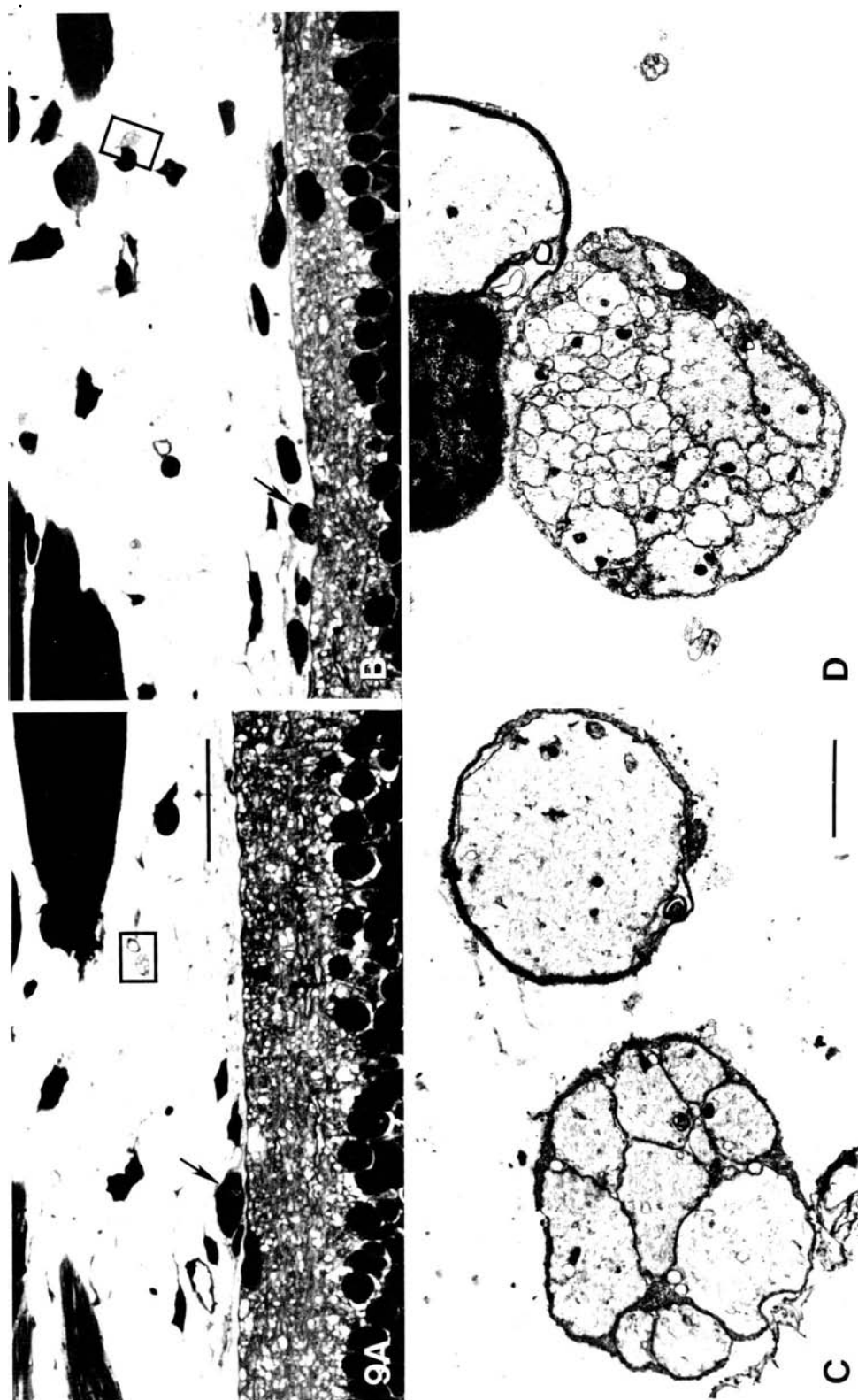


Fig. 9. Light micrographs of horizontal sections through dorsal rami (in boxes) at limb (A) and non-limb (B) levels, of a 21 mm animal, showing the relative sizes of the rami. The ramus size does not usually reflect the number of axons since the axons in limb level rami contain larger diameter axons. This can be seen in electron micrographs (C,D) of the rami (in A,B respectively). The dorsal roots (arrows) are also visible at this level. Scale bars equal (A,C) 100 μm ; (B,D) 2 μm .

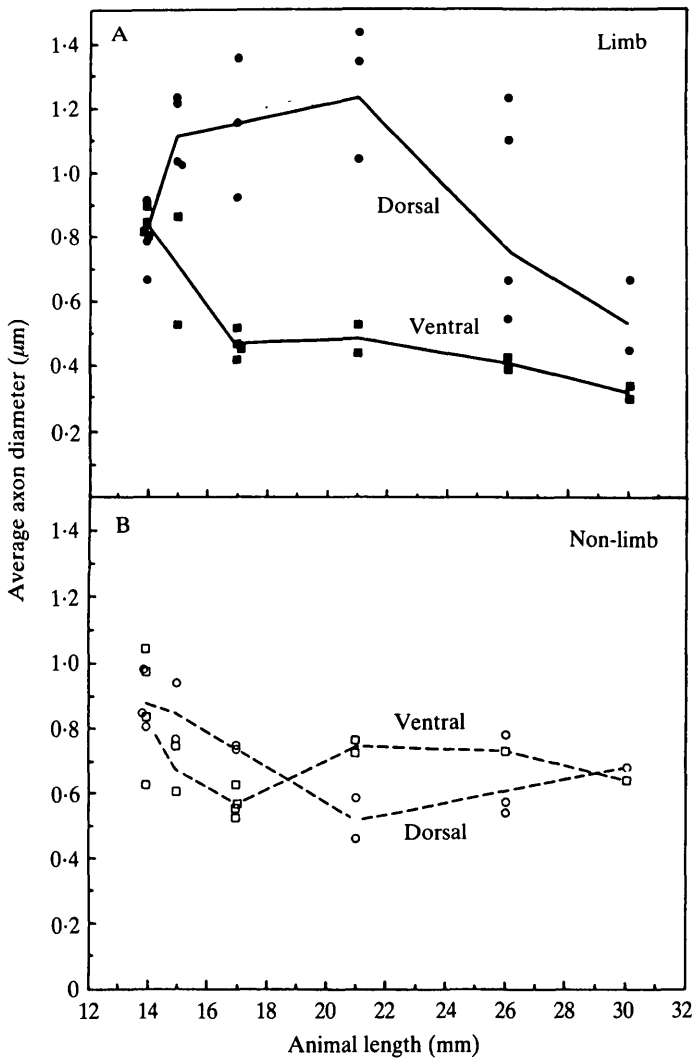


Fig. 10. The average diameter of axons within the dorsal and ventral rami at limb and non-limb levels was determined by pooling data in the same way as described for axon counts (Figs 6, 7). New outgrowth consists of small diameter axons; new outgrowth of axons into established nerves consisting of more mature axons will therefore tend to reduce the average axon diameter. Maturation will tend to increase the average axon diameter. Thus it is possible to see some important developmental events in these data. The average diameters in all four regions are approximately the same in the 14 mm animal but quickly diverge after this developmental stage. (A) The sharp decline in the average diameter of ventral ramus axons at limb levels (■), reflects the onset of extensive new outgrowth. During this same period, dorsal rami axons at these levels (●) increase their diameters as they mature. The disparate values for the 26 mm animal are due to the new outgrowth occurring at level 16 (lower two points) but not yet at level 17 (upper two points). (B) The diameters of dorsal (○) and ventral (□) rami at non-limb levels, reflect the preponderance of outgrowth first into the ventral rami at the 15 mm and 17 mm stages, followed by increased outgrowth into the dorsal rami at the 21 mm stage. Hence the crossover in these two relations and their convergence by 30 mm. This crossover was shown to be significant by applying the Mann-Whitney U-test to the two populations at the 17 and 21 mm stages yielding $P = 0.002$ and $P < 0.001$ respectively.

uncharacteristic of those described in other systems and in the second case they should not properly be regarded as errors.

The comparison of outgrowth at limb and non-limb levels

The size of the body segments diminishes only slightly between limb and non-limb levels. This is reflected in the minimal diminution in axon numbers in the caudal direction from level 20 (Fig. 4). This is also the case in one animal where axons were counted both rostral and caudal to the limb region (Fig. 5). Therefore it seems reasonable to assume in the first instance that axon counts at non-limb levels represent a 'baseline' of neural development associated with trunk innervation, for both 'limb' and 'non-limb' levels. Upon this at limb levels, is superimposed the additional growth associated with the development of the limb. On this basis, the difference between the ventral ramus counts at limb and non-limb levels would indicate the number of axons which innervate the limb.

More importantly, supernumerary axons in dorsal rami at limb, compared to non-limb levels, would indicate that projection errors were being made. This comparison would be particularly sensitive to the incidence of errors at the times of peak outgrowth since even a small number of aberrant axons should appear as a relatively high percentage increase in axon counts in the dorsal rami at limb compared to non-limb levels. This inherent sensitivity is due to the large ventral/dorsal axon ratio, a factor which should be particularly useful in assaying experiments which attempt to produce projection errors in the dorsal ramus.

The results (Figs 4, 5) show that a relative depression arises in axon counts of dorsal rami at limb, compared to non-limb levels. Further examination of the results show that this is due to the failure of any significant new outgrowth to extend down the dorsal ramus at limb levels while outgrowth at non-limb levels increases steadily (Fig. 8B). Hence the exuberant outgrowth occurring at limb levels is exclusively directed into the ventral ramus, and neither random outgrowth nor the extension of collaterals into both rami, appears to occur.

What factors might account for unidirectional outgrowth toward the limb?

There are several possibilities which may account for the unidirectional outgrowth which appears to occur in favour of the limb. Some overriding force may cause dorsal ramus axons to project incorrectly down the ventral ramus. The limb may be capable of remotely exerting such an influence by means of chemotropism (Pollack, Muhlach & Liebig, 1981; reviewed by Berg, 1984) or galvanotropism (Patel & Poo, 1982); both of these mechanisms appear to operate within this range (about 600 μm) *in vitro*. Interestingly, Borgens, Rouleau & DeLanney (1983) have shown that electric currents emanate from the developing axolotl limb bud at a stage which seems to coincide with the period of maximum nerve outgrowth to the limb. The fact that the extent of dorsal ramus 'deprivation' decreases in segments further from the main limb segments (Fig. 5), is also suggestive of a remotely acting attraction which would diminish with increasing distance.

It is also possible that there are physiochemical cues in the rami (reviewed by Carbonetto, 1984) which are confined to limb levels, and which exclusively direct all axons through the ventral ramus, or conversely, produce an effective blockade of the dorsal ramus.

A similar requirement for the active direction of axon outgrowth arises along the ventral rami at limb levels, at points where branches extend into the myotomes. It can be imagined that if limb-bound axons only grow by non-selective fasciculation with existing axons then a significant proportion of them would grow inappropriately into the ventral trunk. This again points to the need for some active process to steer limb-bound axons towards their target. One of the remotely acting influences would tend to have this effect. If a strong non-specific attraction did exist then the ventral trunk may also be 'deprived' of new outgrowth in a similar manner to the dorsal trunk.

The question of whether the limb is capable of influencing the dorsal-ventral distribution is highly amenable to investigation in this preparation, by transplanting limbs to the ventral trunk and/or the ablation of the normal limb buds.

Specificity of connections

The assay described is suitable for the detection of numerical errors rather than errors in specificity. The question remains as to whether neurons are specified at birth to innervate the dorsal trunk, ventral trunk, or limb. Specification of neural populations may of course extend to much smaller groupings. If they are predestined to innervate a certain target area then this raises the possibility that the apparent absence of outgrowth along the dorsal rami at limb levels is a case of errors being made by 'dorsal neurons' projecting down the ventral ramus. This might be in response to the overt influences described above. Alternatively, differentiation of dorsal neurons at limb levels may be simply delayed.

It is interesting to speculate whether or not the transient increase in ventral/dorsal ramus axon ratio at non-limb levels (Fig. 6B), which occurs between 15 and 21 mm stages, is a reflection of the earlier differentiation of 'ventral' neurons (that is, neurons predestined to innervate the ventral trunk). Further tests need to be made regarding this possibility.

Projection strategies at branch points

Developing axons appear to exhibit a variety of responses at branch points, depending on the neural system and the animal. Thus, in the development of rodent retinofugal projections, a highly significant proportion of axons mistakenly project to the contralateral retina (Bunt & Lund, 1981; Sefton & Lam, 1984). Projections of inappropriate axon numbers are also made to the ipsilateral and contralateral thalamus (reviewed by Cowan *et al.* 1984). Thus retinofugal axons extend along all three possible routes at the level of the optic chiasm; collateral formation has been shown not to be a significant process (Jeffery *et al.* 1981).

In contrast, projections from the spinal cord to the limb in the chick appear to be precisely allocated from the beginning (Landmesser, 1980) though experimental

manipulations suggest that these may be passively rather than specifically deployed, at least along the dorsoventral limb axis (Stirling & Summerbell, 1985). In contrast, the frog and axolotl appear to send collaterals down each branch point in the limb plexus, possibly in order to ensure that the appropriate target is contacted without the need for a high degree of specification of the correct route. The emergence of the adult projection may then simply occur by the process of collateral withdrawal. The same animal (this study) appears to direct limb-bound axons into the correct ramus from the outset.

The variety of strategies employed at branch points, even within the same animal, suggests that vertebrates possess a repertoire of mechanisms which enable the specific connectivity of the adult to be achieved.

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