

THE EFFECTS OF NERVE SECTION
AND OF COLCHICINE TREATMENT ON THE DENSITY OF
MECHANOSENSORY NERVE ENDINGS
IN SALAMANDER SKIN

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SUMMARY

We have shown that when one of the spinal nerves supplying the salamander hind limb is cut or treated with colchicine, the fields of the remaining nerves enlarge in area; whereas nerve section produces Wallerian degeneration, the colchicine-treated nerves conducted action potentials normally and their peripheral fields remained unchanged in area (Aguilar, Bisby, Cooper & Diamond, 1973). Since colchicine-treatment reduced neuronal transport, and nerve-section eliminated it, we proposed that nerve sprouting is regulated by factors normally conveyed to the endings by axoplasmic transport.

1. We have now investigated the effects of colchicine on the thresholds and distribution of individual mechanosensory endings in the skin. If reduction of neuronal transport were enough to cause the threshold to be increased to the point of total unresponsiveness, then this could be a sign of an early stage of degeneration in those terminals. It could then be hypothesized that products of degeneration were providing a stimulus for adjacent nerves to sprout.

2. Quantitative physiological studies of the effects of colchicine doses known to interfere with fast axoplasmic transport, indicate that in some experiments the terminal field of the treated nerve was invaded by sprouting fibres from neighbouring axons, when its own endings were unchanged in number, distribution and sensory thresholds. In other experiments the colchicine-treated nerve endings showed an increase in threshold but their function was otherwise unchanged; a similar adjacent nerve sprouting occurred. In a final group, colchicine caused total unresponsiveness of some endings of the treated nerve.

3. When a region of skin was partially denervated by nerve section, the

physiological analysis indicated that the number of new mechanosensory endings which sprouted from the remaining axons exactly matched the number lost by nerve degeneration: furthermore the distribution of the endings was normal. It therefore appears that sprouting ceased when the original density of mechanosensory endings in the skin was restored.

4. The possibility that the drug induced sprouting as a consequence of a direct action on the skin is unlikely. With [^3H]colchicine, we found that the accumulation of label in the skin of the untreated limb, in which sprouting did not occur, equalled that of the opposite limb.

5. The present results lend support to the original hypothesis of Aguilar *et al.* (1973), which proposed that collateral sprouting of intact nerves occurs when the supply of neuronally transported factors becomes inadequate to balance out the effects of a postulated target-tissue stimulus. In the Discussion other examples of collateral nerve sprouting, such as that following adjacent denervation, are shown to be explainable by this hypothesis.

INTRODUCTION

Aguilar *et al.* (1973) proposed that collateral sprouting of normal nerves occurs when a supply of neuronally transported factors becomes inadequate to balance out the effects of a sprouting stimulus which is postulated to be continually released by the target-tissue. The phenomenon of collateral sprouting of intact axons is most easily demonstrated when neuronal transport is totally interrupted in adjacent nerves by surgical section. An alternative explanation for this sprouting assumes that degenerating axons produce a sprouting stimulus, although attempts to demonstrate a stimulating effect of 'products of degeneration' have been unsuccessful (Weiss, 1934; Weiss & Taylor, 1944; but see Hoffman, 1952). We showed previously that colchicine, applied briefly to one of the nerves supplying the salamander hind limb, mimicked the effects of nerve section in that the peripheral fields of the remaining two nerves enlarged in area (Aguilar *et al.* 1973). Although the neuronal transport in the colchicine-treated nerves was reduced, this did not interfere with their ability to conduct action potentials, and they signalled sensory information and drove muscles apparently normally. The mechanosensory fields in the skin were unchanged in area. However, the area of innervation is not a complete measure of the terminal field of the nerve; the *density* of nerve endings could increase or decrease in the absence of an associated change in the area over which they are distributed. This possibility lends itself to a major criticism of our original results, since undetected degeneration of some terminals of the nerve treated by colchicine could provide the stimulus which the traditional explanation of 'denervation-sprouting' invokes.

There is ample evidence that interruption of neuronal transport by nerve section, or by colchicine blockade, causes changes in nerve terminal function before corresponding changes occur in the nerve axons themselves (e.g. Titeca, 1935; Miledi & Slater, 1970; Perisic & Cuenod, 1972). It seems likely therefore that if the nerve terminals in the salamander skin were destined to degenerate, this would be preceded by a stage in which their sensitivity to mechanical stimulation would be altered (such an alteration in threshold however need not necessarily proceed to degeneration proper).

In the preceding paper (Cooper & Diamond, 1977) we analysed the distributions of sensitivities of these mechanosensory endings to obtain a measure of their density in the skin. In the present report we use this information to examine the effects of colchicine on the mechanoreceptor function and distribution, both of the treated nerves and of the adjacent nerves supplying the skin of the salamander hind limb. We have also investigated the possibility that colchicine acts on the skin directly, producing cutaneous nerve sprouting as a secondary phenomenon (cf. Cangiano & Fried, 1977). In addition, we measured the extent to which sprouting occurs after partial denervation of a given region of skin. Our quantitative findings are consistent with our original hypothesis.

Some of the results have been briefly reported (Cooper, Diamond, MacIntyre & Turner, 1975; Diamond, Cooper, Turner & MacIntyre, 1976).

METHODS

All experiments were carried out on adult salamander, *Amblystoma tigrinum*, of both sexes, and of lengths ranging up to 20 cm.

Nerve terminal field measurement

The operative procedures, and measurement for various spinal nerves and their peripheral branches, of the density of mechanosensory nerve endings and the area over which they are distributed, are described in the accompanying paper (Cooper & Diamond, 1976).

Nerve section and colchicine application

The procedures were essentially the same as those described by Aguilar *et al.* (1973). A few mm length of spinal nerves 15, 16 or 17 was exposed near the vertebral column in animals anaesthetized in ethyl-*m*-amino benzoate methane sulphonate (MS 222, Sandoz). When a nerve was sectioned, the central stump was tied off to minimize chances of regeneration. Colchicine was applied by filling the trough made by exposing the nerve (spinal nerve 16) through the overlying muscle. Colchicine (BDH), in concentrations of 50–75 mM, was prepared in amphibian Ringer solution: (NaCl, 111 mM; KCl, 1.9 mM; CaCl₂.H₂O, 1.1 mM; MgSO₄.7H₂O, 1.6 mM, NaHCO₃, 2.4 mM). The bathing period was usually 30 min, after which the solution was washed

out and the wound closed. In some experiments the walls of the trough were coated with petroleum jelly before filling with colchicine solution to reduce systemic absorption of the drug.

[³H]Colchicine experiments

The technique of colchicine application was the same as that described above, except that the applied colchicine was tritiated (colchicine-methoxy-H³(N) (Ring C)) (New England Nuclear, sp. act. 5 c/m-mole) of 40 mc/m-mole final specific activity.

To determine the radioactivity in the skin, the animals were first anaesthetized and decerebrated; dorsal and ventral strips of skin, 6–8 mm wide and 2 mm long, were then dissected from both hind limbs, placed on a small white card, and divided into a proximal and a distal portion. Each of the samples was then prepared for liquid scintillation counting by processing them through a sample oxidizer (Inter-Technique Oxymat). The radioactive ³H₂O was then collected, dissolved in scintillation fluid (composition: 700 ml. dioxane, 300 ml. toluene, 20 g naphthalene, 9 g 25 diphenyloxazole (PPO)), and counted for 10 min in a Beckman LS 230 liquid scintillation counter. The counting efficiencies were calculated by external channels ratios.

RESULTS

Side-to-side symmetry of receptor density

There is no statistically significant difference between the density of the cutaneous mechanoreceptors in the dorsal skin of the two hind limbs in untreated salamanders (see Fig. 4 below). An example of the variability of the density between the two limbs of a single animal is presented in Fig. 1. The histograms show the percent occurrence of given ranges of 'critical stimulus', and as described in the accompanying paper (Cooper & Diamond, 1977), the density of the receptors is indicated by the height of the lowest-threshold bin on the left of the histogram; there is no significant difference between the cumulative frequency plots ($P > 0.2$), i.e. the receptor density is bilaterally symmetrical. Therefore the untreated limb can be used as a control for the treated one.

Effect of interference with neuronal transport on mechanoreceptor function

We have affected neuronal transport in two ways: *nerve section*, which totally interrupts all transport as well as impulse conduction, and *colchicine application* which, as measured by its effect on cholinesterase and catecholamine flow, partially interrupts fast axoplasmic transport, but does not affect impulse conduction (Aguilar *et al.* 1973; M. Holmes, C. Turner, J. A. Fried, E. Cooper and J. Diamond, in preparation). From our previous studies we know that peripheral axons of salamander nerves retain their ability to conduct impulses for 9–12 days after they have been sectioned (Aguilar *et al.* 1973). However, the threshold of the mechanoreceptors is probably a more sensitive indicator of interference with neuronal transport than impulse conduction in the axons (see Introduction).

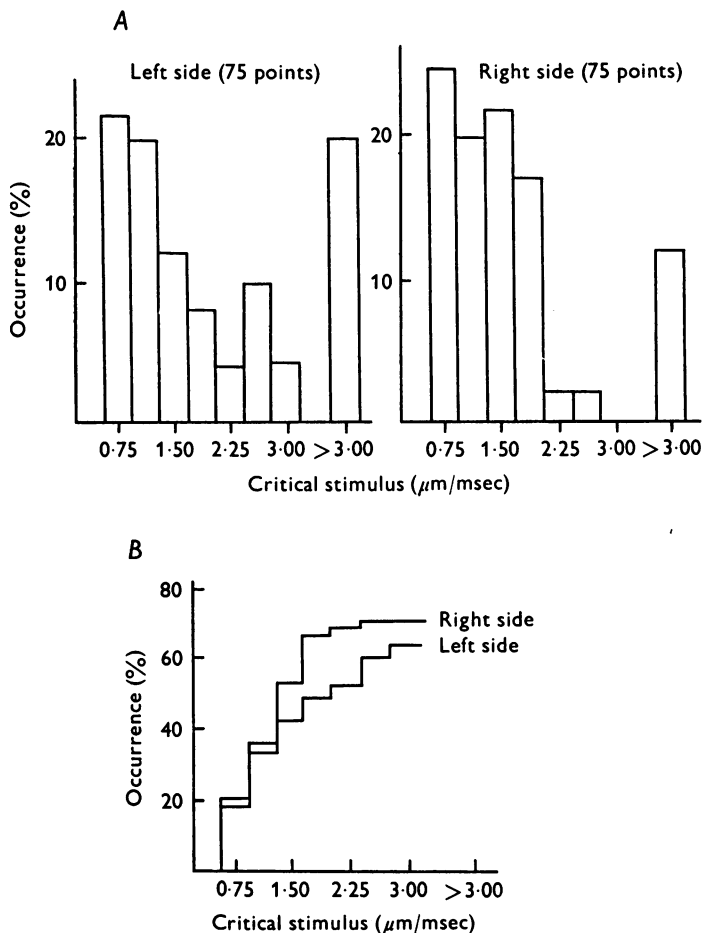


Fig. 1. Comparison of distributions of critical stimulus from one side of the animal to the other.

A, histograms representing the results of 75-point random surveys of critical stimuli in the cutaneous femoris posterior (CFP) nerve fields for right and left hind-limb skin. In this and subsequent histograms the column on the extreme right always represents 'unresponsive' spots, i.e. 'failures', with the range of stimuli we used.

B, the same results are plotted as the cumulative curves corresponding to the histograms. There is no significant difference between the two sides ($P > 0.2$). From the Kolomogorov-Smirnov test, vertical differences $> 26\%$ for a 50-point random survey, or $> 20\%$ for a 75-point survey, indicate that the two comparative curves are significantly different. $P < 0.005$ (see Fig. 2 and text). It should be noted that the difference between these two curves was among the largest we encountered between different regions of normal skin in any one animal. In this and subsequent Figures, the 'failures' are not included in the cumulative curves of % occurrence.

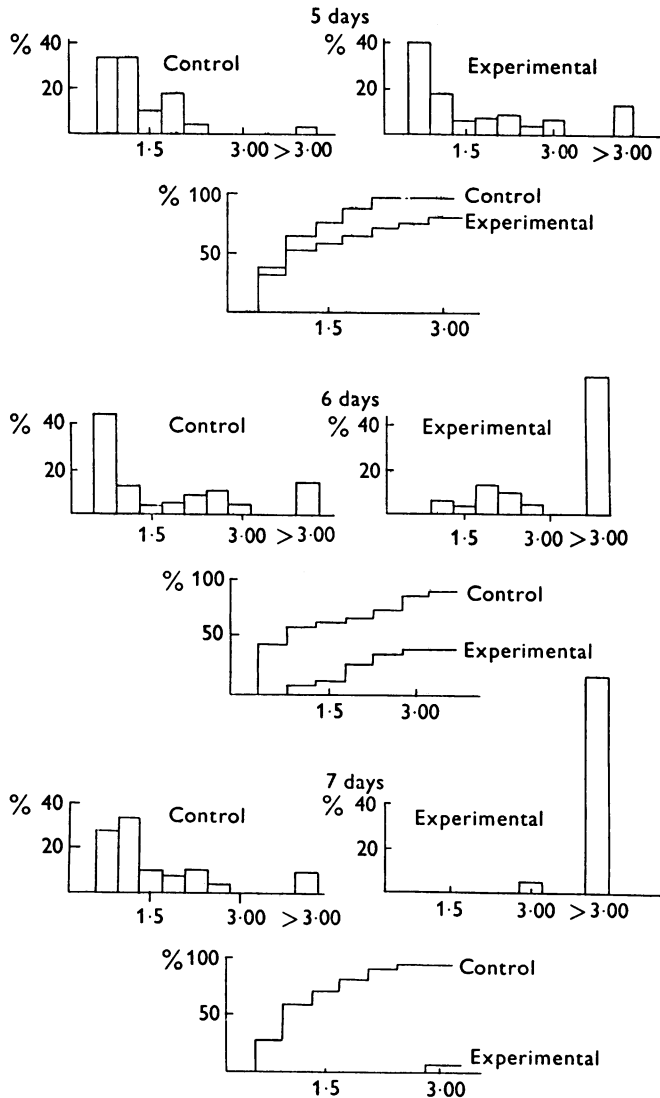


Fig. 2. Mechanosensory function after nerve section. The sensitivity of the 16th nerve field to mechanical stimulation of the skin after 16th nerve section was tested for three different animals, each one examined at a different time (5, 6 and 7 days) after treatment. Impulses were recorded from the distal portion of the sectioned 16th nerve. Each set of three graphs shows the distribution of critical stimuli for a 50-point random survey on the right (experimental) side and on the control 16th field on the other side of the animal, and the corresponding cumulative curves (see Fig. 1). There was no significant difference ($P > 0.2$) between the results on the two sides 5 days after nerve section. However, on day 6 there was a significant change ($P < 0.005$) in the two distributions, and by day 7 only very few skin mechanoreceptors remained functional on the experimental side. Ordinates show percentage occurrence of critical stimuli; abscissae show ranges of critical stimuli used in the 'binning'.

We have found that if the blood supply to the skin is reduced by haemostasis, the threshold of the receptors to mechanical stimulation often rises dramatically within a few hours, and this change in sensitivity is usually irreversible over the period we have followed it (a few hours), even though the axons conduct normally.

We are limited in the extent to which we can measure threshold changes by the maximum stimulus from our crystal driven stimulator; as will be seen below, the bristle used to map the skin provides a considerably greater mechanical stimulus than that supplied by the stimulator, and frequently receptors will respond to the bristle, but are unresponsive as measured by the mechanical stimulator.

Nerve section

Typical results from three different animals, examined (respectively) at 5, 6, and 7 days after the 16th spinal nerve had been sectioned in one limb, are shown in Fig. 2. It can be seen that after 5 days there was no significant difference between the cumulative frequency curves for either hind limb, i.e. there was no functional change in the touch receptors of the sectioned nerve (impulses were recorded from the distal part of the nerve, attached to the skin). In the 6-day animal, however, changes in the distribution of the critical stimulus were clearly apparent; there were fewer low-threshold spots, many more in the higher stimulus range (thereby completely changing the shape of the histogram from its usual skewed distribution), and a significant increase in non-responsive spots. By day 7 about 95% of the touch receptor population was not detectable with the largest stimuli we were able to apply with our mechanical stimulator (Cooper & Diamond, 1977).

Although the length of nerve stump attached to the skin may influence these results (unpublished observations), the time between the first detectable changes, and total unresponsiveness, even to the bristle stimulus, was never greater than 2-3 days.

Colchicine block

In a series of twenty-four animals in which the mechanoreceptors responded apparently normally to the bristle stimulus, approximately half could be recognized as having cutaneous mechanoreceptors whose thresholds were readily detectable as being increased above the normal. These animals were not investigated further, on the assumption that the colchicine treatment might have been so effective as to cause ultimate degeneration of some of the terminals. Our criterion for this rejection was based on the responses of the first fifteen points of the random survey made on the treated side; if ten or more of these were in the high-threshold

range (requiring a stimulus of $2.5 \mu\text{m}/\text{msec}$ or greater) as compared to the normal side, in the skin of which only about three would fall into this category, the experiments were discontinued. In the remaining twelve animals both the experimental and the contralateral control nerve fields were investigated by the routine random survey technique (Cooper & Diamond, 1977).

Fig. 3 shows the results from three animals within the group, examined 6, 9 and 14 days after the 16th nerve on one side had been bathed in a solution of 75 mM colchicine for a period of 30 min. In each of the three animals illustrated there is no significant difference between the results obtained from the two hind limbs; the colchicine treatment therefore did not cause detectable changes in mechanosensory threshold like those observed when the nerve is sectioned (compare Fig. 2). The histograms in these three animals were typical of untreated animals, indicating that colchicine was not having a generalized systemic effect on mechanosensory function.

The method of assessing whether or not colchicine had indeed affected the receptors in this latter group of animals was to compare the distribution of sensory thresholds on the treated and the untreated sides of the animal, using the Kolmogorov-Smirnov test; these distributions are bilaterally symmetrical in normal animals (see Fig. 1). Briefly the Kolmogorov-Smirnov test states (in the context of this testing situation) that if there is a difference in % occurrence of more than 20 at any given stimulus on the cumulative frequency curve, then for a 75-point survey one side is significantly different from the other at the $P < 0.005$ level. For ten of the twelve animals in the latter group there was no significant difference between the mechanosensitivity of the skin between the two sides as tested in this way.

We tested the possibility that colchicine caused a *consistent* shift of receptors from the low threshold range in this group of animals. We compared the mean ratio of the side-to-side % occurrence in this low-threshold range, for all twelve animals in this group (including the two which *were* shown to have differences by the Kolmogorov-Smirnov test), to the mean ratio similarly obtained for a group of untreated animals. The results are shown in Fig. 4. It is apparent that there is a wide scatter in both groups, although there is no significant difference between them ($P > 0.05$). For there to be a significant effect of colchicine ($P < 0.005$) the mean ratio (E/C) in the experimental group would need to be 0.55, assuming the same scatter. We have determined that the average value of % occurrence of low-threshold receptors for normal skin is 29 ± 3.6 . Therefore, for a reduction in the mean ratio to 0.55, colchicine would have to reduce this value of % occurrence by about 13. This is a more sensitive

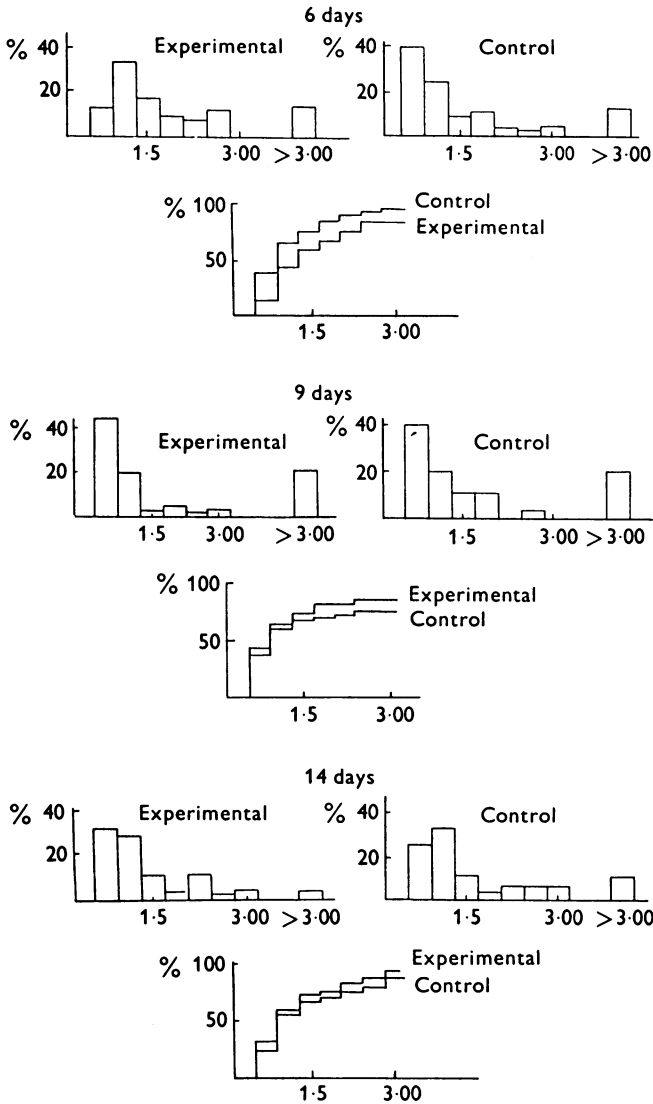


Fig. 3. Mechanosensory function after colchicine. After one 16th nerve trunk was treated with colchicine, the sensitivity of both 16th nerve fields to mechanical stimulation was tested for three different animals, each one examined at a different time (6, 9 and 14 days) after treatment. The distributions of critical stimuli, and the cumulative curves, for 50-point random surveys is compared. There was no significant difference ($P > 0.1$) between the results for the two sides. Ordinates, % occurrence of critical stimuli. Abscissae, ranges of critical stimuli. Compare these results with those of Fig. 2.

test for an adverse effect of colchicine than the Kolmogorov–Smirnov one on the individual animals, since the latter requires a drop of 20 in the % occurrence to be significant. When there *is* a consistent change in the value of the low-threshold bin, it is readily detected by this method, as seen in the example of sprouting after partial denervation (see Fig. 10). (An advantage of the Kolmogorov–Smirnov test is that it reveals shifts in threshold over the entire range used, and not only the low-threshold one).

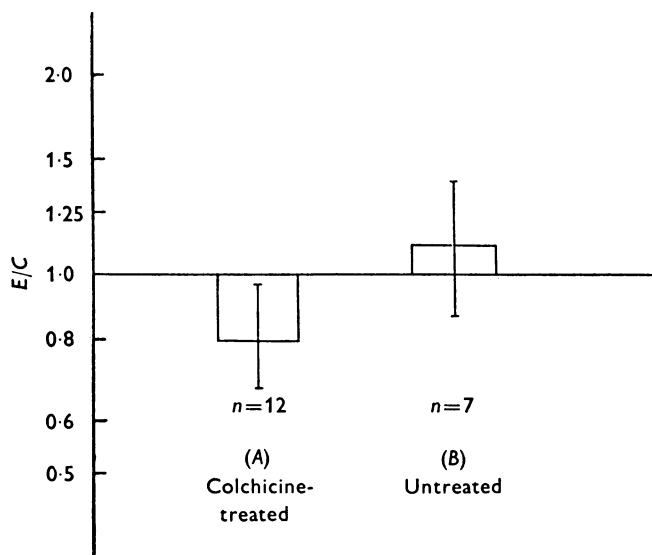


Fig. 4. Low-threshold receptor density after colchicine treatment. In twelve animals the 16th nerve was treated with 50–75 mM colchicine for half an hour, and 6–21 days later recordings were made from the same nerves while testing the mechanosensitivity of the appropriate skin field. There was no significant difference ($P > 0.05$) between the mean ratio of the side-to-side % occurrence of low-threshold spots for the colchicine-treated group to the mean ratio, similarly obtained for an untreated control group of animals. The ordinates, E/C , represents the ratio of occurrence of low-threshold spots in the experimental (E) and control (C) sides. For the untreated group E and C were right and left sides. Note the values of E/C represent the geometric means and therefore are plotted on logarithmic scales (see text).

On the basis of all our findings in this type of experiment, we conclude that our technique of colchicine application, and the dosage we used (which we know affects neuronal transport), was able in many animals to leave the receptor threshold and density essentially unchanged. The strength of this conclusion must be seen in relation to other conditions, such as nerve section or anoxia, in which changes in mechanosensory function were

strikingly affected at a time when axonal impulse conduction and excitability were unimpaired.

The group of colchicine treated animals dealt with in this section was that from which the examples given below of adjacent nerve sprouting without changes in function of treated nerves were taken.

*Effects of interference with neuronal transport in
nerve 16 on the adjacent nerve fields*

Density and area of nerve fields

In our original studies we assessed sprouting by measuring the extension of nerve fields 15 and 17 into that of field 16. Such an extension only occurs if fields 15 and 17 do not already abut upon each other (Diamond *et al.* 1976). In the animals we now use the fields of 15 and 17 usually do share a common frontier, and field extension only rarely can be exploited;

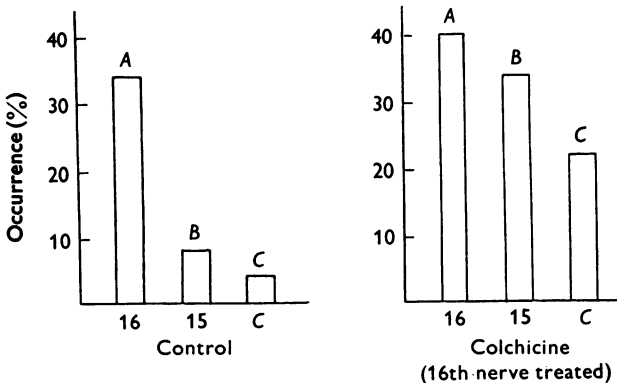


Fig. 5. Sprouting after colchicine treatment. In a single animal the touch receptor density (i.e. the % occurrence in the 'low-threshold' range; see text and Fig. 7 of Cooper & Diamond, 1977) in a region of skin shared by the 15th and the 16th nerve was investigated after the 16th nerve on one side was treated with colchicine. There was no loss in the population of receptors feeding into the untreated 16th nerve, compared to the control 16th nerve (column A). In this animal the number of touch receptors associated with the 15th nerve was only a small proportion of the total. However, on the treated side the 15th nerve now supplied an extra population of receptors almost equal to the number associated with the 16th nerve (column B). The third column, C (referred to as coincidence) indicates the amount of overlap between the fields of the low-threshold receptors (see text).

measurements of receptor density have to be employed. In most of the experiments now to be described the principle measurement of sprouting will be an increase in receptor density.

(i) *Hyperinnervation in absence of changes in treated population.* Fig. 5

illustrates an experiment in which neither the thresholds nor the density of the endings of the colchicine-treated 16th nerve were changed, and shows the % occurrence of lowest-threshold spots. In this animal the 16th and 15th nerve fields overlapped, but less than a third of the receptors in the common field were those of the 15th nerve. However, 3 weeks after the colchicine application there was a marked increase in the number of low-threshold spots supplied by the 15 nerve (the apparent increase in those of the colchicine-treated 16th nerve was not statistically significant). This

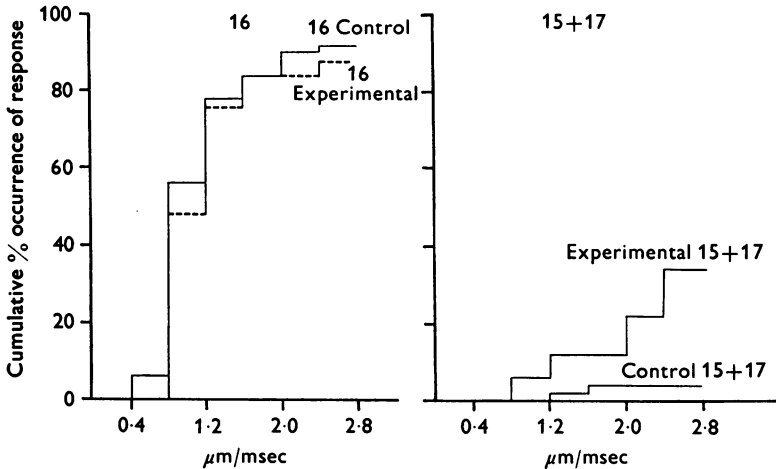


Fig. 6. Cumulative frequency curves of the treated (right) and untreated (left) 16th nerve receptors, and of the combined 15th and 17th receptors on the right and left sides. There is no significant difference between the 16th nerve populations on the two sides ($P > 0.2$). But the difference between the total population belonging to the 15th and 17th nerves was significantly greater on the right than on the left (see text).

result indicates that new 15th nerve endings were hyperinnervating the field of the 16th nerve, whose own endings were still functioning normally. A striking indication of the 15th nerve sprouting was the increase in the number of spots which showed 'overlap' i.e. at which the prodger evoked a low-threshold response in both the 15th and the 16th nerve (Fig. 5). In normal skin the number of sensitive spots which are supplied by more than one axon is rarely greater than 5% of the total (cf. Cooper & Diamond, 1977).

Fig. 6 shows a rare example in which the region of skin investigated had an overlap of all three spinal nerves, 15, 16 and 17. The 16th nerve on the right side had been treated with colchicine 21 days earlier, and there is no significant difference between the cumulative frequency plots for the

two 16th nerves on both sides; i.e. the colchicine had not affected the function or number of 16th nerve endings. The 15th and 17th mechanosensory nerves have been considered together as the 'adjacent' (untreated) nerve in this experiment. When the combined receptor population of these nerves is compared between the two sides, there is a significant increase in that of the treated limb. Fig. 7 illustrates another example of increase in untreated nerve receptor population at a time when that of the treated nerve was unchanged by the colchicine treatment.

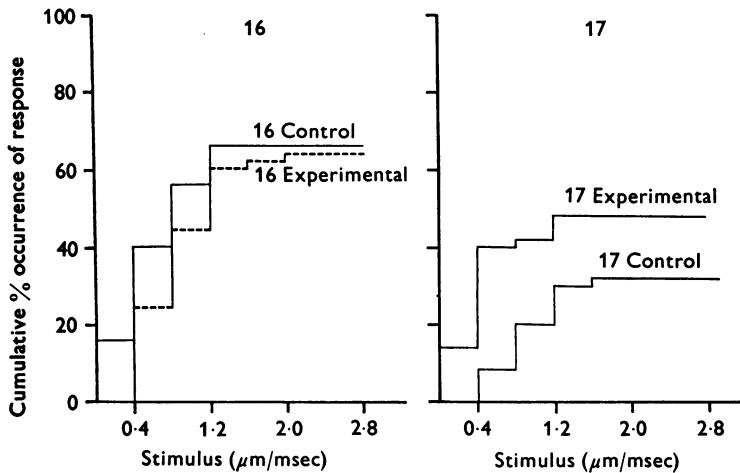


Fig. 7. Cumulative frequency curves for the right and left 16th nerve mechanoreceptor populations, and for the right and left 17th populations. The difference between the two 16th nerve populations is not significant ($P > 0.2$). The difference between the two 17th populations is significant ($P < 0.005$).

(ii) *Area sprouting without apparent increase in density.* In the experiment of Fig. 8 we were fortunate in that on the colchicine-treated side the 15th nerve field had extended into that of the treated 16th nerve, whose density and thresholds of endings were quantitatively unchanged. Obviously this result implies an overall increase in the density of endings in that field. However, the 15th fields were mapped with the bristle technique. When we used the crystal stimulator to identify single receptive points, only those of the 16th nerve were low threshold. The cumulative frequency curve indicated that the newly sprouted 15th nerve endings had high thresholds (Fig 8B). For a few of these sites we attempted to locate single receptors by systematic plotting of the sensitive regions, and found them to be genuinely high threshold (cf. Fig. 12, Cooper & Diamond, 1977). This showed directly that the result was not explainable by a

failure to 'hit' a low-threshold spot in a region where there was simply a very low density of newly sprouted 15th nerve receptors of normal threshold.

(iii) *Hyperinnervation with changes in receptors of treated nerve.* In some experiments colchicine produced some drop-out, or increase in threshold beyond the range of the stimulator, of receptors belonging to the treated nerve; since not all the receptors were affected, it was impossible to identify those that were still functional when tested by the bristle stimulus. However, the sprouting of the other nerves was more than

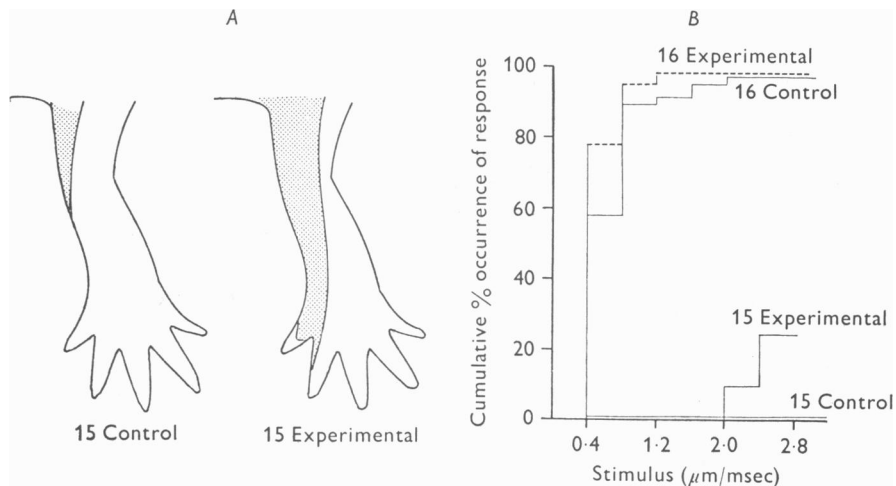


Fig. 8. *A*, fields of the right and left 15th nerves. On the right side the 16th nerve had been treated with colchicine 3 weeks earlier. The increase in the 15th field is striking. The fields of the 16th nerve are not indicated, but in both limbs they included the entire dorsal surface, which is that shown.

B, cumulative frequency curves for the right (experimental) and left (control) 16th nerve receptors and for the 15th nerve receptors on right side. These results are from the same animal represented in part *A*. There was no significant difference between the two 16th mechanoreceptor populations ($P > 0.2$). Although there were no 15th nerve low-threshold receptors on the right side in the extended field of the 15th nerve (see Fig. 8*A*), high-threshold ones can be seen in the histogram, and in the field they were detectable readily by the bristle stimulus. On the control side there were no 15th nerve receptors in the corresponding region (see Fig. 8*A*, and also Fig. 9, and text).

compensated for by this change; this is a significant finding, and is in contrast to the result obtained when neuronal transport was *totally* interrupted in the 16th nerve by sectioning (see below). In the experiment of Fig. 9 we were again dealing with a limb in which the sprouting response of the 15th nerve had resulted in an increase in the area of its field; at the time of the mapping the newly-sprouted receptors had higher-than-normal thresholds. From the histograms it can be seen that the number of unresponsive 16th nerve spots on the treated side (right) was significantly greater than the control; the simplest explanation of this is that the colchicine had caused a

functional drop-out of some receptors, although, as in the previous example, we cannot be sure that they would not all have responded to the bristle stimulus. However, as the histograms of the 15th nerve show, in the region investigated there were no 15th nerve endings on the control side, but on the experimental side receptors were now present which, though of high-threshold, *more than compensated for* the fall in the number of 16th nerve receptors. The histograms for the right 15th

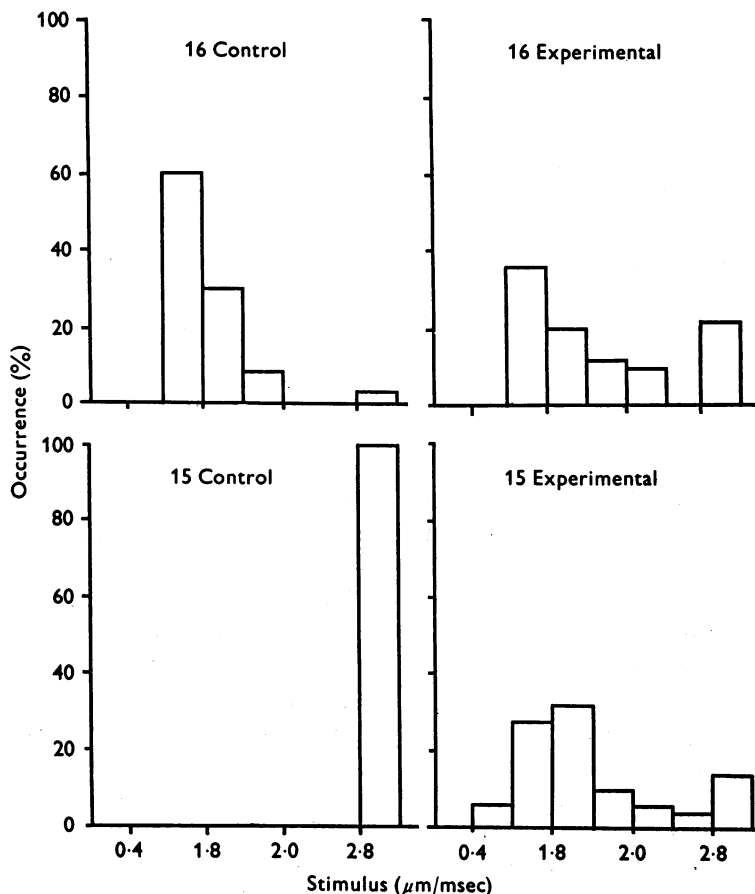


Fig. 9. Percentage occurrence histograms for the 15th and 16th right and left nerves. The 16th right (experimental) nerve had been treated with colchicine some 21 days before the quantitative investigation. There was a significant drop-out of receptors feeding into this nerve (compare 'failure' columns, which are those on the extreme right of each histogram). There were no 15th nerve receptors at all on the left (control) side in the field investigated (i.e. 100% 'failures') but on the right side there had been extensive sprouting which was revealed as an occurrence of mainly high-threshold receptors. Note the difference between the shape of the histograms of the 16th and 15th nerves on the experimental side. The latter histogram is that of the distribution of a variable-threshold population.

nerve show an abnormal distribution in which the highest threshold bin is not on the left; this is a typical distribution of a variable-threshold population in which most of the receptors were high threshold. In fact, the number of normal low-threshold 15th nerve receptors which appeared actually matched that of the 16th nerve which had been lost. From other findings we can assume that in time most of the high-threshold receptors would have 'matured', and a normal-threshold hyperinnervation of the skin would have resulted.

Effects of total interruption of neuronal transport by nerve section

In these experiments the 16th nerve was cut at least 3 weeks before the subsequent investigation of density of endings in the region of the skin supplied exclusively by the CFP nerve (Cooper & Diamond, 1977). Fig. 10 shows the results from a group of nine animals, in which there was no increase in *area* of the 17th nerve; it compares the density of cutaneous mechanoreceptors (given by the % occurrence of low-threshold responses in the random survey) on the right (experimental) with the left (control) side. It can be seen that after total elimination of the 16th nerve contribution to the CFP field, a large increase occurred in the number of low-threshold receptors supplied by the 17th nerve. The mean value of these on the operated side was more than double that on the control, although there was a wide variation. This variation depends largely upon the relative number of 16th and 17th axons present in the CFP nerve; in some animals 16th spinal nerve axons supplied the majority of endings to the area, in others the 17th. In the former group, elimination of the 16th fibres resulted in large increase in the population of 17th nerve endings, while in the latter situation only a relatively small increase in 17th nerve endings was observed. Nevertheless in these summed results, the over-all increase in 17th endings is clear. The middle column (*B*) of Fig. 10 expresses the receptor population on the experimental side as a fraction of that on the control side. The experimental side was supplied by only one nerve, the 17th, and this gave rise both to the 17th component of the original population of receptors, plus all the newly sprouted ones. The control limb receptors were supplied by both the 16th and the 17th nerve. There is no significant difference ($P > 0.2$) between the side-to-side density of cutaneous mechanoreceptors in these experimental animals as compared to the group of untreated animals shown in column *C*. This result indicates that the number of new sprouts on the experimental side had quantitatively made up the number of receptors in which function had disappeared as a consequence of nerve section.

The histograms of distribution of 'critical stimuli' over the region of the skin in which sprouting had occurred were no different from those in the control limb. This indicates that the newly sprouted endings not only matched the *number* of those which had been lost, but their *thresholds* were

not significantly different from those of the original receptors, and their distribution was identical with the distribution of the total (16th and 17th) endings which had previously existed in the skin.

Sprouting, or unmasking of 'silent' endings?

In a few experiments in which enlargement of the *field areas* of the 15th or 17th nerves occurred after section or colchicine treatment of nerve 16, we investigated the possibility that a 'fringe' of functionally silent endings around the measured fields had become sensitive to mechanical stimulation, so giving an appearance of sprouting of the original nerves within the field. Normally there is a high degree of symmetry of corresponding fields between the two high limbs. Therefore we could assume that a

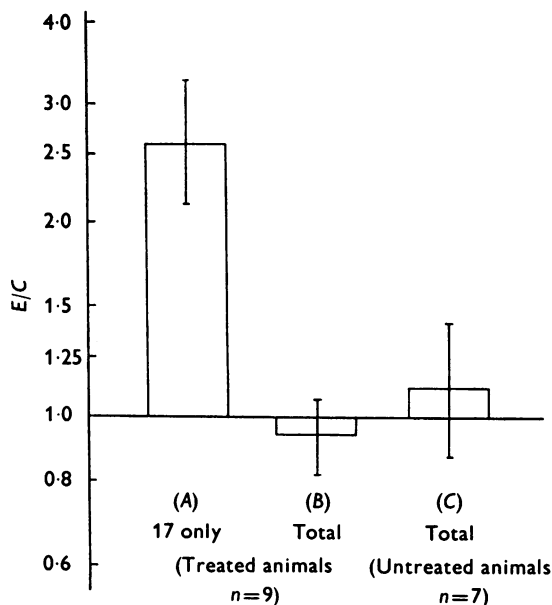


Fig. 10. Quantitative sprouting after partial denervation. The % occurrence of low-threshold receptors feeding into the 16th and 17th nerves from a shared region of skin was measured, and the values compared between right and left limbs. Column *A* refers to a group of animals in which the right 16th nerve had been sectioned three weeks previously, and shows the right (*E*)/left (*C*) ratio for the number of 17th nerve touch receptor population only. An increase in 17th nerve receptors is clearly seen. Column *B* shows, for the same group of animals, the right-left ratio for the total receptor population (that is the 17th on the treated side, the 16th plus the 17th on the control). Column *C* shows right-left ratios for the total population of touch receptors in a control group of animals, with 16th plus 17th nerves intact on both sides. There is no significant difference ($P > 0.2$) between column *B* and column *C*, indicating that the increase in 17th nerve receptors on the right side of the experimental group had quantitatively made up the loss due to 16th nerve section (vertical bars equal s.e. of mean). Note *E/C* represents the 'geometric mean' (cf. Fig. 4).

postulated non-functional fringe would be similarly present on both sides. We used a fine needle electrode to apply localized electrical pulses to the skin. Within the field of a nerve these pulses readily excited one or more single receptive spots (presumably the individual axonal branches associated with single mechanoreceptors). Immediately outside the mapped field, even very large pulses (e.g. approximately four times greater than those which were effective within the field) failed to excite impulses in the nerves supplying the mapped field. From the results it was clear that if there were 'silent' endings (to mechanical stimulation) then their axons were electrically inexcitable.

We conclude that sprouting of new endings is the obvious explanation for the field enlargement we measured.

Investigation of possible direct action of colchicine on skin

These experiments were designed to test whether or not there was preferential accumulation of label on the treated side after tritiated colchicine application to the 16th segmental nerve. Such an accumulation could suggest a direct effect on the skin which might produce nerve sprouting as a secondary phenomenon (cf. Cangiano & Fried, 1977; Speidel, 1941).

The amount of labelled material in the skin was measured by scintillation counting, and assuming no degradation of colchicine or loss of label is expressed as μmole colchicine per mm^3 of skin. Sixteen animals were used, all receiving colchicine on the same day. Two of them were investigated on each successive day, beginning on the day after treatment, for 7 days; two were investigated at 14 days after treatment. The results are presented in Table 1.

There were no significant differences ($P > 0.2$) between amounts of labelled material in the skin on one side of the animal compared to the other. Furthermore there is little difference in the skin counts for one day animals compared to the animals examined 14 days after treatment. The variation in the amount of radioactivity between animals is not surprising considering the method used to apply the drug to the nerves, which involves no special precaution to restrict colchicine only to the nerve.

DISCUSSION

Hypothesis of collateral sprouting

We have proposed that collateral sprouting is initiated by a target-tissue stimulus, whose effects are regulated by neuronally transported factors; these factors are presumed to be continually released from the nerve endings (Aguilar *et al.* 1973). Our original evidence for this hypothesis was that colchicine, appropriately applied to one of the nerves supplying the salamander hind limb, could mimic the effects of nerve section by causing sprouting of the adjacent nerves, both in skin and in muscle. Electrophysiological and morphological investigations of the axons of

TABLE 1. Colchicine in skin after application to one 16th nerve. The values in this table are the number of moles of colchicine $\times 10^{-8}$ per mm^2 of skin for different animals treated. Skin thickness averages $300 \mu\text{m}$. The calculations assumed that the colchicine was not degraded

| Days after treatment | Animal | Dorsal skin | | Ventral skin | |
|-------------------------|---------------------------|-----------------|-----------------|-----------------|-----------------|
| | | prox. region | dist. region | prox. region | dist. region |
| 1 | $^3\text{HC-6}$ <i>E</i> | 4.35 | 3.81 | 4.38 | 4.38 |
| | <i>C</i> | 5.07 | 4.26 | 4.32 | 4.41 |
| | $^3\text{HC-5}$ <i>E</i> | 6.72 | 6.66 | 5.88 | 5.97 |
| | <i>C</i> | 6.36 | 5.34 | 5.25 | 5.40 |
| 2 | $^3\text{HC-23}$ <i>E</i> | 4.50 | 3.85 | 3.66 | 4.26 |
| | <i>C</i> | 4.35 | 3.78 | 4.05 | 4.20 |
| | $^3\text{HC-12}$ <i>E</i> | 4.41 | 3.93 | 3.45 | 3.42 |
| | <i>C</i> | 4.77 | 3.87 | 4.47 | 4.62 |
| 3 | $^3\text{HC-9}$ <i>E</i> | 1.98 | 1.74 | 2.04 | 1.74 |
| | <i>C</i> | 2.19 | 1.89 | 1.56 | 1.47 |
| | $^3\text{HC-26}$ <i>E</i> | 4.83 | 3.72 | 4.62 | 4.26 |
| | <i>C</i> | 5.13 | 4.26 | 4.68 | 4.29 |
| 4 | $^3\text{HC-25}$ <i>E</i> | 1.95 | 1.89 | 1.89 | 1.74 |
| | <i>C</i> | 2.16 | 2.19 | 1.98 | 1.95 |
| | $^3\text{HC-34}$ <i>E</i> | 3.66 | 3.30 | 3.66 | 2.88 |
| | <i>C</i> | 2.79 | 2.67 | 3.21 | 2.67 |
| 5 | $^3\text{HC-31}$ <i>E</i> | 3.15 | 3.03 | 3.87 | 4.08 |
| | <i>C</i> | 3.15 | 3.15 | 3.30 | 3.21 |
| | $^3\text{HC-39}$ <i>E</i> | 5.61 | 4.71 | 5.40 | 4.47 |
| | <i>C</i> | 4.92 | 4.62 | 4.83 | 4.92 |
| 6 | $^3\text{HC-48}$ <i>E</i> | 3.72 | 3.51 | 2.82 | 2.85 |
| | <i>C</i> | 3.66 | 3.21 | 3.03 | 2.73 |
| | $^3\text{HC-46}$ <i>E</i> | 5.13 | 4.92 | 3.30 | 2.61 |
| | <i>C</i> | 4.50 | 3.93 | 3.03 | 3.21 |
| 7 | $^3\text{HC-47}$ <i>E</i> | 4.47 | 3.63 | 3.66 | 3.00 |
| | <i>C</i> | 3.84 | 3.09 | 2.61 | 3.15 |
| | $^3\text{HC-40}$ <i>E</i> | 3.99 | 3.72 | 3.72 | 3.63 |
| | <i>C</i> | 3.90 | 3.45 | 3.72 | 3.93 |
| 14 | $^3\text{HC-10}$ <i>E</i> | 2.79 | 2.25 | 2.40 | 2.16 |
| | <i>C</i> | 2.37 | 2.31 | 2.52 | 2.25 |
| | $^3\text{HC-19}$ <i>E</i> | 5.04 | 4.20 | 3.93 | 3.42 |
| | <i>C</i> | 4.92 | 4.14 | 4.05 | 3.57 |
| Mean \pm s.e. of mean | <i>E</i> | 4.14 ± 0.33 | 3.66 ± 0.30 | 3.66 ± 0.27 | 3.42 ± 0.27 |
| | <i>C</i> | 3.96 ± 0.30 | 3.51 ± 0.24 | 3.57 ± 0.27 | 3.51 ± 0.27 |
| | | $P > 0.2$ | $P > 0.2$ | $P > 0.2$ | $P > 0.2$ |

the treated nerves showed them to be essentially normal (except for the reduced number of microtubules in the axons). However, we did not exclude the possibility that colchicine may have caused a scattered degeneration of some of the endings of the treated nerve. An objection to our hypothesis could then be that this degeneration might have provided some products which could then be postulated as responsible for sprouting. Such terminal degeneration could have occurred without necessarily proceeding into the parent axon; the axons conducted impulses normally and did not have signs of Wallerian degeneration. If such scattered degeneration of terminals occurred, it did not in fact alter the area of the field of the treated nerve. A second objection to our original evidence is that colchicine may have acted directly at the level of the target-tissue, and caused sprouting of nerves as a secondary phenomenon. Cangiano & Fried (1977) have shown that colchicine appears to have an action on muscle which mimics some features of denervation; this action of colchicine is independent of the ability of the drug either to interrupt axoplasmic transport, or to cause muscle inactivity by interfering with neuromuscular transmission in some other way.

The findings with the ^3H -labelled colchicine indicate that it does not cause sprouting as a secondary effect of a direct action on the skin. Whether the label which accumulates in skin is colchicine or some degradation product, we can be certain from our results that it occurs equally on both sides of the animal, and therefore does not account for a unilateral sprouting response.

Quantitative evidence for role of neuronal transport

Total interruption of neuronal transport by nerve section causes the threshold of the mechanosensory endings in salamander skin to rise within about 5 days, and most endings become unresponsive to mechanical stimulation in a further 2–3 days. We have shown that sprouting of adjacent nerves is detectable by approximately 5 days after nerve section (Aguilar *et al.* 1973). Although colchicine treatment yielded various results, in a number of these quantitative experiments there was no significant change in the density and threshold of the mechanosensory endings of the treated nerve, over the periods we investigated. Nevertheless sprouting of adjacent nerves occurred, and the skin became hyperinnervated. These particular results are difficult to reconcile with the principal argument against our hypothesis.

In some experiments the histograms of critical stimuli showed an increase in the number of high threshold responses for the colchicine-treated nerve. The Kolmogorov-Smirnov test showed that in these animals there was no reduction in the total number of mechanoreceptors, but only a shift to a higher threshold of a proportion of them. There were other experiments (e.g. that of Fig. 9) in which some endings

became unresponsive to our technique of mechanical stimulation, although we could not be sure that they would have failed to respond to the bristle stimulus; we presumed therefore that we had on occasions achieved a partial 'chemical' denervation, possibly confined to the terminal region of some of the colchicine-treated axons. These changes probably indicate that the colchicine penetrated into some axons to a greater extent than others, and consequently caused a greater reduction in the neuronal transport of the former. Axons would be expected to be affected to a different extent by our colchicine treatment, the outermost receiving a higher concentration than those at the core of the nerve trunk. The doses of colchicine and the bathing technique we used in the present experiments were adequate to interfere with fast transport (Aguilar *et al.* 1973), and as we have since quantified, slow flow continues at the time that fast transport is significantly reduced in these nerves (M. Holmes, C. Turner, J. A. Fried, E. Cooper and J. Diamond, in preparation).

In the experiments in which transport was totally interrupted by nerve section, the new sprouts quantitatively made up in number those which had disappeared; however, they did not exceed that number (see below). Those experiments in which threshold changes could be detected in a proportion of the endings of the colchicine-treated nerves gave additional evidence supporting our general hypothesis. If these affected endings are considered to be similar to those whose parent axons had been sectioned, we would expect new sprouts to arise in a quantity which would match this number of affected endings. However, the actual number which appeared sometimes *exceeded* the number of affected endings. The extra population of untreated nerve endings most probably results from a reduction of neuronal transport in colchicine-treated axons whose endings were functionally unaffected; this would be in accordance with the experiments in which the endings of the colchicine-treated axons were unchanged. It is not surprising that there were experiments in which threshold changes occurred in the colchicine-treated nerve endings in animals in which there was adjacent sprouting. There are no *a priori* grounds to suppose that any one consequence of a reduced neuronal transport (adjacent sprouting) would be apparent at an earlier time than another (e.g. changes of sensory thresholds).

We have evidence (unpublished) that newly-sprouted endings may take about 3 weeks to 'mature' to the normal low-threshold state. The results of experiments like that shown in Fig. 8 suggest that such maturation may not be completed, at least during the first 2-4 weeks after sprouting was initiated. These high-threshold endings were responsive to the bristle stimulus, but could not be excited by the prodder. We are investigating this phenomenon further. It is possible, however, that in those experiments in which we failed to detect an increase in density by the prodder technique, sprouting of such high-threshold receptors had occurred. These experiments in which no increase in density was observed were on animals in which enlargement of field area by sprouting could not occur (Diamond *et al.* 1976).

Our analysis assumes that low-threshold mechanosensory endings have similar receptive fields (see Cooper & Diamond, 1977). By 'sprouting' we mean the production of more such endings, not an enlargement in the sensitive area of individual

pre-existing ones. Is it possible that the colchicine-treatment causes degeneration of some of the treated nerve endings, with *enlargement of the area of the receptive fields* of the remaining endings, and of those of adjacent nerves? This seems unlikely. New 'endings' certainly take more than two weeks to mature to the level which can be detected by our stimulator. But ones destined to degenerate (e.g. after nerve section) are readily recognized by a functional 'drop-out' in less than 8 days, and by an increased threshold even before this. Even if 'colchicine death' were a slower process than that following nerve section, it would be unlikely to extend the time to appearance of altered endings by more than 2-3 days (cf. Singer & Steinberg, 1972). In our survey of mechanosensory thresholds of colchicine-treated nerves, we would therefore have seen threshold changes in affected terminals at a time clearly separated by some 5-6 days at least, from the time when an apparent 'recovery' occurred (in other endings of the treated nerve). We obtained no evidence of any such phenomenon, although the period up to 3 weeks after treatment by colchicine was that with which we were especially concerned.

In addition, the analysis of the radius and spacing of individual mechanoreceptive fields (Cooper & Diamond, 1977) which we made on normal skin, we also occasionally made on skin supplied by colchicine-treated nerves, and the results were essentially normal, with no evidence of unusually large receptive fields of widely separated low-threshold receptors.

Finally, we showed (Aguilar *et al.* 1973) that colchicine-treated nerves are unable to respond by sprouting when adjacent nerves are sectioned, suggesting that it would be unlikely for some of the endings to be killed by colchicine while others of the same treated nerve would respond by sprouting or 'enlarging'.

Our results give support, some of which is quantitative, for our hypothesis for collateral nerve sprouting, which suggests that the reason why nerves sprout when adjacent ones are sectioned is because of the interruption of neuronal transport in the cut nerves. We find no compelling reason to suppose that such 'denervation sprouting' depends upon the effects of degeneration products released from the cut nerve.

The stimulus for sprouting

If neuronally transported factors regulate collateral sprouting, what supplies the stimulus to the nerves to induce sprouting? We suggest that the target tissue is in fact the source of such a stimulus. An analogous situation exists with the respect to nerve growth factor and its action on sympathetic and sensory nerves, at least during early development (Levi-Montalcini & Angeletti, 1968; Zaimis, 1972; Stöckel & Thoenen, 1975). According to our hypothesis an equilibrium state exists in the periphery; the effects of the target-tissue sprouting stimulus are balanced by those of neural factors released from the nerve endings, constituting a negative feed-back control mechanism. If then some nerve axons are sectioned there are at least two situations possible whereby the balance could be restored. One would be that more of the neural factor would be released by the remaining nerve endings; the other would require that the rate of release of neural factor is constant, and that sprouting would continue

until the original number of endings, and presumed release sites, would be restored. Our results strongly support the latter interpretation. After sectioning one nerve in a shared area of skin, the sprouting of the remaining nerves continued until the original density of endings was re-established. Sprouting then ceased. Furthermore, our analysis of the distribution of critical stimuli (Cooper & Diamond, 1977) showed that the spacing of newly sprouted endings was very similar to that normally found in the skin. One interpretation of this would be that the new endings occurred at or very near to the sites from which endings had been lost as a consequence of a nerve section.

The mechanosensory endings are fairly uniformly spaced out in salamander skin (Cooper & Diamond, 1977); this is consistent with our hypothesis of an equilibrium existing between a sprouting stimulus and an inhibitory neural factor released from the endings. Such an interaction might also explain the finding that, in general, each axon 'owns' an exclusive region of skin (cf. Cooper & Diamond, 1977).

Other examples of collateral sprouting

We have elsewhere discussed our new hypothesis in the context of nerve sprouting as a general phenomenon (Diamond *et al.* 1976). For example, Olson & Malmfors (1970) found that a nerve-free piece of iris transplanted into the anterior chamber of the rat eye, caused sprouting of the undamaged sympathetic fibres of the host, clearly suggesting the presence of a target-tissue stimulus. There is sprouting of botulinum-treated motor nerves at muscle in the absence of visible nerve degeneration (Duchen & Strich, 1968); we hypothesize that in addition to preventing the release of acetylcholine, the toxin also prevents the release of the regulatory neural factors. Ramon y Cajal (1919) pointed out that sprouting of nerves during primary development occurs only when the nerves arrive at their target-tissues, in the case of epithelium at least; his suggestion to explain this phenomenon is analogous to our hypothesis, namely that a sprouting stimulus derives from the target-tissue, and becomes eventually neutralized by factors released from the nerve. The findings of Fitzgerald (1961) on the development of the innervation of the pig snout suggest that a target-tissue can be matched throughout its growth by an appropriate increase in the nerve endings without a change in the number of parent axons; the density of innervation stays constant. More target-tissue would presumably make more sprouting stimulus, so inducing sprouting until the equilibrium state is reached.

Following monocular deprivation earlier in life either by eye removal (Wiesel & Hubel, 1974) or by lid suture (D. Hubel, T. Wiesel and S. Le Vay, personal communication), lateral geniculate neurones connected to one

eye in monkeys enlarge their territories in layer IV at the expense of adjacent territories associated with the other eye. Obviously there could be selective sprouting of one set of terminals in this instance at the expense of those of the other; the deprived lateral geniculate cells may be reduced in size, and could well have a reduced neuronal transport (cf. Grafstein, Murray & Ingolia, 1972).

The control we have described in this report is particularly concerned with the regulation of the *density* of endings. In another report (J. Diamond, and D. MacIntyre, in preparation; see also Diamond *et al.* 1976) we describe a spatial influence which seems more to control the area over which the nerve terminals sprout. The implications of the existence in the mature animal of mechanisms which serve to regulate the density (and area) of nerve terminal fields would seem most important in the central nervous system. It is of great interest, therefore, that collateral sprouting of intact axons occurs in the regions of partial denervation in the mammalian brain, and that these sprouts can make functional synapses (see Diamond *et al.* 1976, for review and discussion).

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