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# ON THE PREDOMINANTLY SINGLE INNERVATION OF SUBMANDIBULAR GANGLION CELLS IN THE RAT

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SUMMARY

1. Simultaneous intracellular recordings were made from pairs of submandibular ganglion cells to examine why each of these neurones is generally innervated by a single preganglionic axon.

2. Impalements made within isolated clusters of two to fifty cells showed that a preganglionic axon typically innervates several neurones within a group.

3. The neurones innervated by a particular axon tended to be intermingled with other neurones; some neurones were innervated by an axon that was not shared by any of their immediate neighbours.

4. These results indicate a mechanism that causes a preganglionic axon to innervate exclusively many of the neurones it contacts, presumably by successful competition with nearby axons that initially innervate the same cells and continue to provide innervation to neighbouring neurones.

5. Re-innervation of adult ganglion cells shows that this mechanism persists or can be reactivated in maturity.

## INTRODUCTION

The submandibular ganglion of the rat presents a striking example of the phenomenon of 'synapse elimination' during normal development: each ganglion cell is innervated at birth by an average of five different preganglionic axons, while in maturity each is generally contacted by a single axon (Lichtman, 1977). The relatively simple structure of this ganglion provides an opportunity to study which of several different arrangements of preganglionic axons results in singly innervated ganglion cells. For example, singly innervated ganglion cells might occur because (1) each preganglionic axon innervates only one ganglion cell, (2) each preganglionic axon innervates a group of adjacent cells or (3) each preganglionic axon innervates a number of ganglion cells intermingled with neurones innervated by other axons, analogous to the arrangement of motor units (Edström & Kugelberg, 1968; Burke & Tsairis, 1973; Burke, Levine, Salcman & Tsairis, 1974). The purpose of the present work was to distinguish between these possibilities. The results show that the distribution of neurones innervated by the same preganglionic axon (a neural unit) is in fact similar to the arrangement of muscle fibres in a motor unit in that the ganglion cells innervated by a particular axon are distributed amongst cells innervated by other axons. This implies a competitive mechanism by which most or all of

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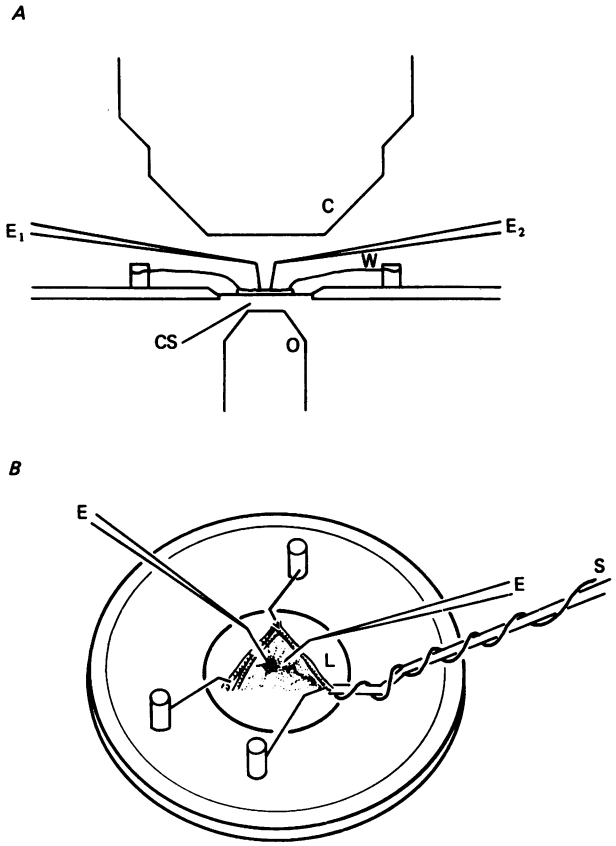



Fig. 1. Chamber used for intracellular recording from the submandibular ganglion. *A*, side view of apparatus. Ganglia were mounted on a 0·1 mm thick coverslip (CS), pinned flat with stainless steel wires (W), and viewed with an inverted differential interference contrast microscope (O, objective) (Biovert, Reichert). A pair of microelectrodes ($E_1$, $E_2$) were positioned over the ganglion using two independently controlled micromanipulators. The electrodes were bent near their tips to allow easy movement within the working distance (7 mm) of the microscope condenser (C). *B*, top view of recording chamber and ganglion. A suction electrode (S) was applied to the lingual nerve (L) just proximal to the ganglion for stimulation.

the synaptic sites on a ganglion cell become innervated by the same preganglionic fibre in spite of innervation by other axons during early post-natal life and their continued proximity in adult ganglia.

## METHODS

Thirty-five adult albino rats (Sprague–Dawley females, 160–220 g) were anaesthetized with chloral hydrate (0·35 gm/kg, I.P.) and both submandibular ganglia removed and maintained in oxygenated Ringer fluid at room temperature. The methods of dissection, nerve stimulation and the criteria of the satisfactory impalement of ganglion cells have been described previously (Lichtman, 1977).

The ganglia were pinned out in a small perfusion chamber and viewed with differential interference-contrast optics (Fig. 1). The submandibular ganglion consists of a number of neuronal clusters, some of which lie in a thin connective tissue sheet between the lingual nerve and the

salivary ducts (see Pl. 1, for example, and Lichtman, 1977). The sizes, shapes and positions of these isolated groups are variable, as are the number and positions of the preganglionic bundles which enter the ganglion from the lingual nerve. Sixty clusters in forty ganglia were studied; each cluster consisted of less than fifty cells and was generally one cell in thickness.

The preganglionic axons were stimulated by applying a close fitting suction electrode to the cut end of the lingual nerve. The number of axons innervating a ganglion cell could then be estimated by counting the number of discrete steps in the excitatory post-synaptic potential (e.p.s.p.) in response to graded stimulation of the preganglionic nerve. Although generally reliable, estimates of the number of fibres innervating a neurone obtained by this method are subject to several uncertainties. For example, if a fibre making a large synaptic contribution were activated at a low intensity of stimulation, a small synaptic potential from another axon with a higher threshold could be obscured because of shunting. That this sometimes occurred was evident from antidromic stimulation of fibres making *en passant* synaptic contacts. A further difficulty with this method was that the latency of e.p.s.p.s. often decreased as the strength of stimulus increased. Presumably this occurred because the action potential was initiated closer to the point of recording. As a consequence, changes in latency were not used as a criterion of additional recruitment. The size of the e.p.s.p. was also estimated by measuring the amplitude of the synaptic potential timed to occur during the refractory period of a directly elicited action potential (Purves, 1975).

To determine if two neurones were innervated by the same axon, pairs of ganglion cells were monitored simultaneously while stimulating the lingual nerve. Neurones were considered innervated by the same preganglionic axon if post-synaptic potentials elicited by lingual nerve stimulation (1) appeared in both cells at the same stimulus strength, (2) had the same latency and (3) always failed together when the stimulus strength was adjusted so that the preganglionic axon fired about half the time. The over-all innervation of groups of cells was examined by maintaining the impalement of one neurone as a reference while other neurones in the same cluster were successively impaled with a second micro-electrode. Each pair was then tested to see if the same preganglionic fibre innervated both cells. In this way, even cells not examined simultaneously could be shown to be innervated by the same axon (see Pl. 1 for example). By using several different reference cells and re-impaling some neurones, seventeen groups of two to eighteen cells were completely analysed: in these clusters the number of innervating axons as well as the number and position of cells innervated by each axon were known (see Pl. 2 for example). A less complete analysis of innervation was made in forty-two larger clusters (of ten to fifty-two cells). In all these experiments neurones were identified and labelled on Polaroid photographs of the clusters at 630 ×. This method of estimating the number of neurones innervated by an axon underestimates the true size of neural units because only cells in a particular cluster are counted. Since many ganglion cells show a synaptic response to antidromic stimulation of the nerves running distally along the salivary ducts, preganglionic axons almost certainly innervate neurones other than those in the particular clusters studied (see Lichtman, 1977).

Although cells included in this study had action potentials of at least 60 mV upon initial impalement, the action potential amplitude or re-impaled cells was sometimes lower. However, synaptic potentials in re-impaled cells were still apparent, and thus the criteria described above could still be applied.

To see whether the predominantly single innervation of ganglion cells was restored after re-innervation, the submandibular ganglion was denervated in thirty-nine additional rats by crushing the chorda tympani near the medial side of the sphenoid spine. Crushing the chorda tympani effectively denervated the ganglion as stimulation of the lingual nerve elicited no synaptic potentials in 137 of 139 ganglion cells impaled within 10 days of crushing. Denervated and contralateral control ganglia were then studied at intervals of up to one year after the nerve crush.

## RESULTS

### *Intracellular recording from individual ganglion cells*

Graded stimulation of the lingual nerve in adult animals elicited a single step in the post-synaptic response of 607 (78 %) of the 776 neurones impaled in this study. One hundred and fifty-six ganglion cells (20 %) showed two steps in the post-synaptic

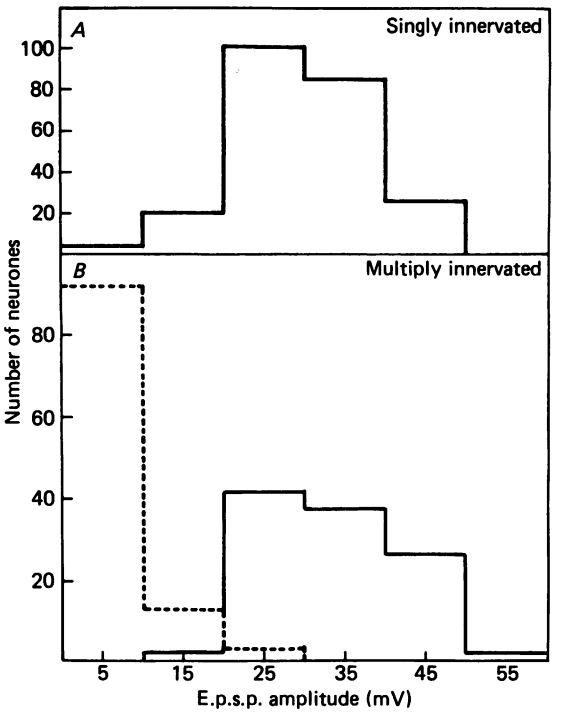
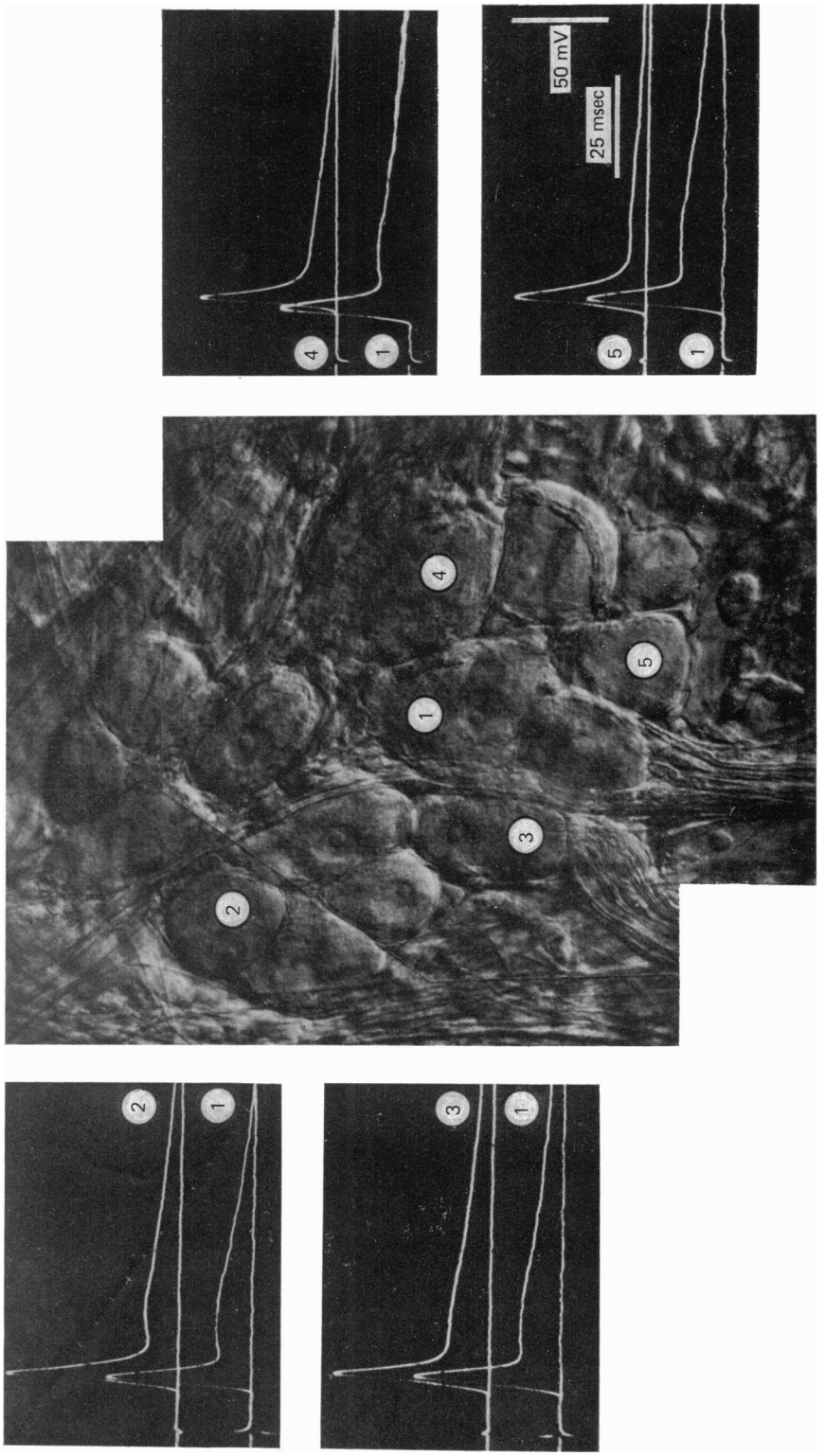



Fig. 2. Histograms of the mean e.p.s.p. amplitudes of primary and secondary inputs to submandibular ganglion cells. In most cells graded stimulation of the lingual nerve elicited a single synaptic potential; in multiply innervated cells one or more smaller (secondary) potentials were also evident. *A*, the distribution of synaptic potentials in 236 singly innervated cells. Nearly all of these cells (94 %) were driven to threshold by lingual nerve stimulation. *B*, distribution of synaptic potentials in multiply innervated cells. E.p.s.p. amplitudes in 108 cells in which the secondary potential (dashed line) could be elicited at a lower stimulus strength than the primary input (continuous line). Only 7 % of the secondary potentials were above threshold. The amplitude distribution of the primary synaptic potentials in multiply innervated cells was generally similar to that in singly innervated cells, although these measurements in the multiply innervated cells represent the depolarization resulting from the activation of both primary and secondary inputs.

potential and 13 (2 %) had three steps. Most of the cells showing multiple steps in their synaptic response could be fired by only one of the innervating axons (subsequently called the primary input; Fig. 2); only 12 of the 169 multiply innervated neurones (7 %) were brought to threshold by two axons, and none by three. Thus, in confirmation of an earlier study (Lichtman, 1977), the majority of adult ganglion cells are innervated by a single preganglionic axon, and even those that are multiply innervated receive a strong synaptic input from only one axon.

*Simultaneous recording from pairs of ganglion cells*

To test whether individual preganglionic axons provide innervation to more than one ganglion cell, pairs of neurones located in the same cluster were monitored simultaneously with intracellular electrodes during lingual nerve stimulation. In


50 mV
25 msec
4
1
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1

217 (43 %) of 500 pairs from sixty clusters, both cells were innervated by a common axon. Thus, preganglionic fibres frequently innervate more than one cell in a cluster.

Adjacent neurones within a cluster were somewhat more likely to be innervated by the same axon than cells located at some distance from one another: 56 % of the adjacent neuronal pairs were contacted by the same axon, while the same axon innervated 38 % of non-adjacent cell pairs. Although this difference is significant, nearly half (44 %) of the adjacent ganglion cells were not innervated by the same axons. Since these neurones are closely packed (see Pl. 1, for example) and receive most of their innervation on or near the cell bodies (Lichtman, 1977), the absence of common innervation to many adjacent cells is surprising.

### *The over-all innervation of clusters of neurones*

The innervation of forty ganglion cell clusters was further analysed by maintaining the impalement of one ganglion cell as a reference while a second micro-electrode was used so impale other cells in the group successively. In the example shown in Pl. 1 (see opposite), one electrode remained in a reference cell (1) while the other electrode successively impaled cells 2, 3, 4 and 5. The upper left oscillograph shows simultaneous intracellular records of cells 1 and 2 during lingual nerve stimulation. At one stimulus strength neither cell fired, while a small increase in the stimulus elicited suprathreshold responses in both cells (two traces are superimposed in each of the records shown). Moreover, the responses always failed together when the stimulus was adjusted to threshold levels, and had nearly identical latencies. Thus these cells were innervated by the same axon. The electrode in cell 2 was then used to impale cell 3 while the other electrode remained in the reference cell (1) (lower left oscillograph). As with the initial pair, both cells showed synaptic potentials at the same stimulus strength, failed together when the stimulus was adjusted to threshold levels, and had nearly identical latencies. Accordingly, cells 1 and 3 were also innervated by the same axon, and, even though they were not monitored simultaneously, cell 3 must have been innervated by the same axon as cell 2. The electrode in cell 3 was then used to record from cell 4. Cells 1 and 4 were not innervated by the same axon since at one stimulus strength cell 1 gave a synaptic response while cell 4 did not; furthermore, when the stimulus strength was increased a post-synaptic potential was elicited in cell 4, but its latency was different from the response elicited in cell 1 (upper right oscillograph). Finally, cell 5 was impaled while the reference electrode remained in cell 1, and was found to be innervated by the same axon that innervated cells 1, 2 and 3 (lower right oscillograph). By continuing to impale different cells in the group with one electrode while the other remained in the reference cell (1), the number and position of all the cells in the group innervated by the axon innervating cell 1 could be determined.

By using several different cells as references it was possible to analyse completely the innervation of some clusters. Thus in seventeen additional clusters of two to eighteen

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EXPLANATION OF PLATE 1

Determination of the number and positon of cells innervated by a particular axon within a cluster. See text above for explanation. Calibration bar = 20 $\mu$m.

cells (ten on average) the total number of innervating axons, and the positions of the cells innervated by each axon, were determined (Fig. 3 and Pl. 2). These groups were usually innervated by about four to five axons (range one to ten) and each axon contacted an average of two to three ganglion cells (range one to nine). No strict regional organization was apparent in the innervation of ganglion cells within these clusters. In fact, thirty-four of the 170 cells (20%) were not adjacent to any cells innervated by the same preganglionic axon (see, for example, Pl. 2*D* and *E*). Thus

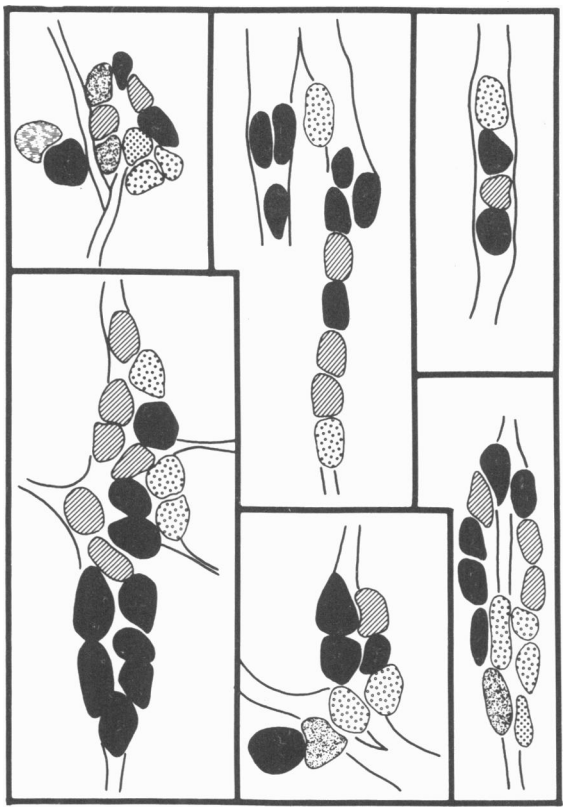

Fig. 3. Camera lucida drawings showing the arrangement of preganglionic innervation in six different neuronal clusters. Cells innervated by a particular axon in each cluster have been given the same shading. Only the primary innervation of each cell is represented.

individual neurones could not only be adjacent to cells receiving different innervation but could even be entirely surrounded by cells innervated by axons different from those providing their own innervation.

The intermingling of neural units within clusters was typical. In several groups, however, the cells innervated by one axon were grouped together (see Fig. 3), and in three of the seventeen clusters all of the cells were innervated by the same axon; the largest of these clusters contained thirteen cells.

About 22% of the ganglion cells studied received one or more weaker (secondary) inputs in addition to innervation from a primary axon (see Fig. 2). One explanation

of this multiple innervation might be that a preganglionic axon providing the primary innervation to a ganglion cell also establishes a few synapses on adjacent cells. However, more than half of the multiply innervated cells (twenty-one of thirty-six) in the eighteen completely analysed clusters were not adjacent to any cell innervated by the axon supplying their secondary innervation. Thus a neurone's secondary innervation does not necessarily arise from spillover of the innervation to immediately adjacent cells. This result also argues against the possibility that apparent multiple innervation might result from the diffusion of transmitter from endings on adjacent neurones.

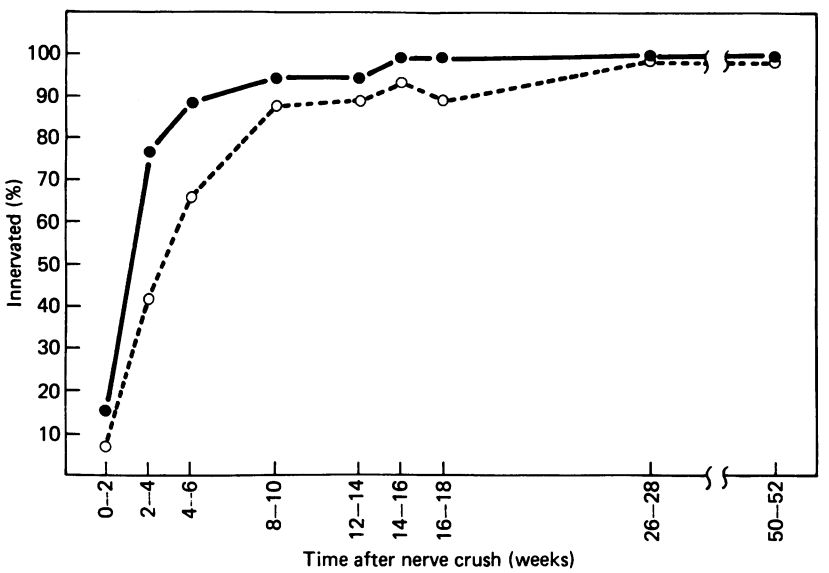


Fig. 4. Time course of the reinnervation of submandibular ganglion cells following crush of the preganglionic chorda tympani nerve. Solid line shows the proportion of cells in which there was some innervation; broken line represents suprathreshold innervation. By 6 months nearly all of the reinnervated neurones could be driven to threshold. Each point represents 35 to 139 neurones (see Fig. 5).

In summary, preganglionic axons may innervate all, or nearly all, of the postsynaptic sites on ganglion cells without innervating immediately adjacent sites on neighbouring cells. Thus some mechanism permits a preganglionic axon to distinguish the sites on the cells it ultimately innervates from nearby sites on cells innervated by other axons.

*Re-innervation of the submandibular ganglion*

To examine whether the mechanism that gives rise to single innervation persists in adult animals, ganglia were denervated by crushing the preganglionic chorda tympani nerve, and examined at intervals of up to a year (Fig. 4). Some re-innervation was observed as early as 10 days after the crush, and within one month, 75 % of the cells were re-innervated to some degree (40 % of the re-innervated cells received only subthreshold innervation at this stage). Two to six weeks after nerve crush a

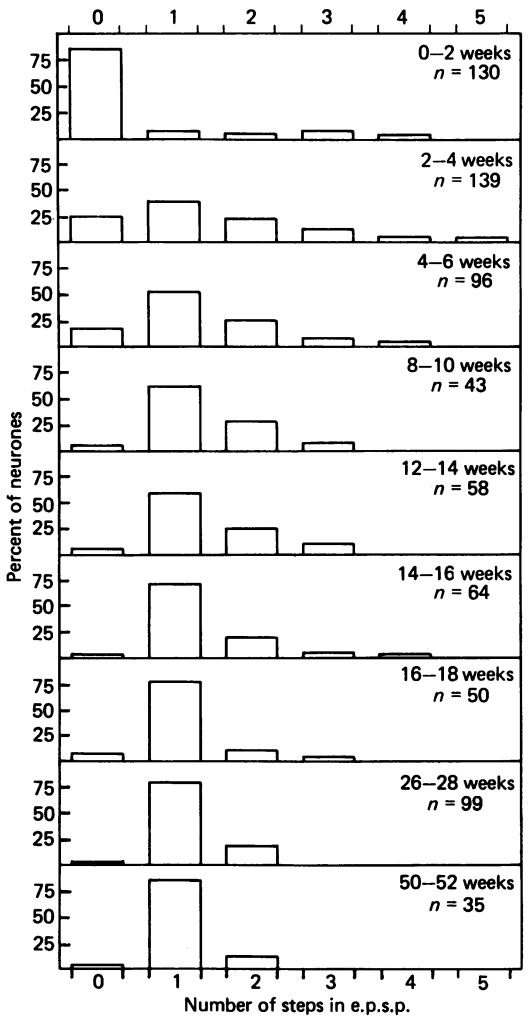


Fig. 5. Histograms of the number of fibres innervating ganglion cells estimated from the number of steps in the synaptic response recorded at intervals after chorda tympani crush (O = uninnervated cells). During the early course of re-innervation more neurones than normal showed multiple steps in their synaptic response; by 16–18 weeks, however, about 80 % of the cells were singly innervated, and only 20 % multiply innervated. This ratio of single to multiple innervation after 16 weeks is about the same as that found in normal animals and in the contralateral control ganglia (4:1).

greater than normal number of cells showed multiple steps in the synaptic response to graded stimulation of the lingual nerve (Fig. 5): 48 % showed more than one e.p.s.p. step compared to 23 % on the control side (and 22 % in unoperated animals). The proportion of multiply innervated ganglion cells gradually decreased: 6 months after preganglionic nerve crush re-innervated and contralateral control ganglia had about the same percentage of multiply innervated cells and showed no further change a year post-operatively. A similar result has been obtained in studies of the reinnervated mammalian neuromuscular junction (McArdle, 1975; see also Tate & Westerman, 1973, and Jansen & Van Essen, 1975).

The decrease in the number of axons innervating ganglion cells between the second and eighth week of re-innervation occurred despite an increase in the percentage of cells which could be driven to threshold by preganglionic stimulation (compare Figs. 4 and 5). This suggests that more new synapses were being formed during this period than were being lost through the elimination of multiple innervation.

These results show that the mechanism that gives rise to the innervation of all, or nearly all, of the synaptic sites on a ganglion cell by one axon persists or is reactivated in adult rats (see however, Ko & Roper, 1978; Dennis & Sargent, 1978).

## DISCUSSION

The major question addressed by these experiments is why cells in the submandibular ganglion are singly innervated. The results rule out two possible explanations: (1) that cells are singly innervated because each preganglionic axon innervates only one ganglion cell and (2) that ganglion cells are singly innervated because each neuronal cluster is innervated exclusively by one axon. On the contrary, individual preganglionic axons usually (perhaps always) innervate more than one ganglion cell, and the cells innervated by one axon are generally intermingled with cells innervated by other axons.

The innervation of the submandibular ganglion is similar in several respects to the innervation of skeletal muscle. For example, in both systems reorganization of synaptic connexions during development gives rise to predominantly singly innervated cells (Redfern, 1970; Bennett & Pettigrew, 1974, 1975; Brown, Jansen & Van Essen, 1976; Lichtman, 1977). A further similarity between the innervation of muscle and the submandibular ganglion is that cells innervated by the same axon are intermingled with cells innervated by different axons (Edström & Kugelberg, 1968; Burke & Tsairis, 1973; Burke *et al.* 1974). Thus in neither system do individual presynaptic axons innervate regions of the target exclusively, although axons have a strong tendency to capture individual cells more or less completely.

The mechanism that accounts for the innervation of individual submandibular ganglion cells by one axon almost certainly involves competition, since polyneuronal innervation in neonates gives way to innervation by a single dominant axon during the first few weeks of post-natal life. Considering the proximity of the post-synaptic sites on adjacent cells, the number of preganglionic axons in the vicinity of each cell, and the fact that about five axons innervate each post-synaptic cell during development, the failure of many adjacent neurones to be innervated by the same axon is remarkable. As there is little reason to believe that the submandibular ganglion cells within a cluster differ qualitatively from one another, selectivity is an unlikely basis for the unitary capture of neurones. Rather the competition seems to be quantitative in the sense that it is more concerned with the single innervation of each ganglion cell than with which axon innervates a particular cell (see Purves & Lichtman, 1978, for further discussion). Although it is not known what preganglionic axons compete for, the implication of the unitary capture of ganglion cells by preganglionic axons is some form of positive feed-back that reinforces the synapses an axon makes on a particular cell without necessarily sustaining the synapses made by the same axon on adjacent cells.

I wish to thank Dr Dale Purves for his invaluable advice and continued interest in all phases of this work. I am also grateful to Dr C. C. Hunt for the loan of the differential interference contrast microscope and to Drs Hunt, C. M. Rovainen, W. Thompson and E. Rubin for helpful criticism. This work was supported by U.S.P.H.S. Grant NS 11699 to Dr Purves, a grant from the Muscular Dystrophy Association of America and N.I.H. Training Grant 5T32 GM7200.

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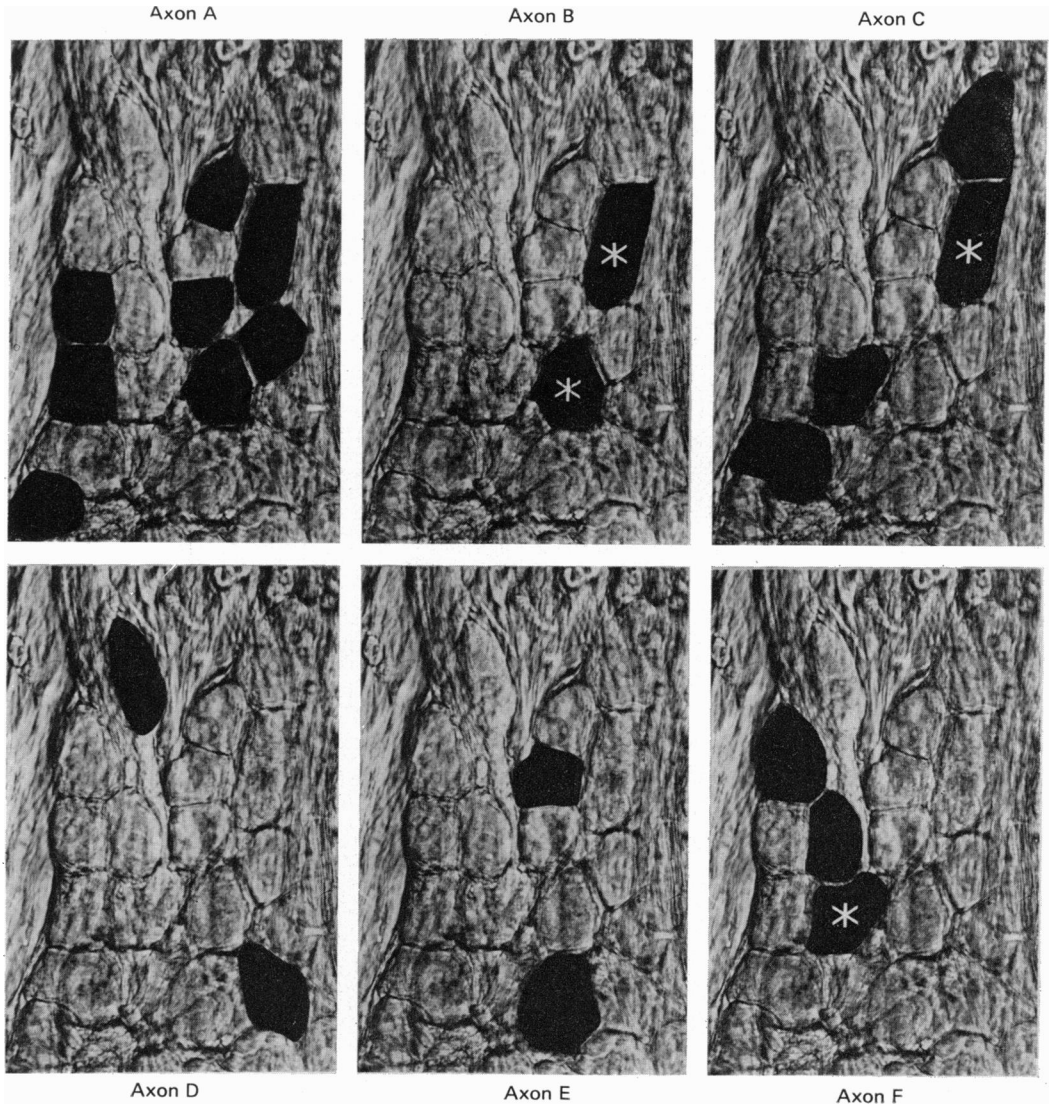
## EXPLANATION OF PLATE 2

The innervation of a completely analysed cluster of seventeen neurones. Each frame shows the cells innervated by one of the six axons which contributed innervation to the group as a whole. Cells marked with an asterisk received a secondary input from the axon shown in that frame. Calibration bar = 20 $\mu$m.

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