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DESTRUCTION OF A SINGLE CELL IN THE CENTRAL NERVOUS SYSTEM OF THE LEECH AS A MEANS OF ANALYSING ITS CONNEXIONS AND FUNCTIONAL ROLE

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SUMMARY

A method has been devised for killing an individual neurone in the C.N.S. of the leech by injecting it with Pronase. The technique has been used to examine the role of individual sensory and motor cells involved in producing reflex movements.

1. After a neurone was injected with Pronase, either in an intact animal or an isolated ganglion, its cell body lost its resting and action potentials. Some hours later the injected cell's axons in the periphery failed to conduct impulses. In the intact animal the cell body could no longer be discerned after a few weeks.

2. To test for destruction of processes within the neuropile, cells were injected first with the enzyme horseradish peroxidase (HRP) and then several hours later with Pronase. Absence of the characteristic HRP reaction product indicated that Pronase had spread throughout the arborization of the cell.

3. Injection of Pronase into one cell did not produce overt electrophysiological or anatomical changes in other cells in the ganglion including neurones that were originally electrically coupled to the killed cell.

4. Evidence that an individual cell was the only motoneurone supplying particular muscles was provided by destruction of that cell in otherwise intact animals, which resulted in a characteristic motor deficit in the area supplied by the killed cell. Over a period of months, functional recovery of the affected muscles occurred by way of homologous cells in adjacent ganglia.

5. A further application of the technique was to trace the connexion that a particular sensory neurone makes onto two motoneurones that are electrically coupled. Normally, the sensory neurone gives rise to excitatory potentials in both post-synaptic cells. Synaptic potentials could still be recorded in one motor cell after the other had been destroyed by Pronase, indicating that synapses were made directly onto both of the motoneurones.

INTRODUCTION

In the relatively simple nervous systems of invertebrates a single neurone or groups of a few neurones often perform functions equivalent to those requiring large numbers of cells in the vertebrate nervous system. This has made it possible to use intracellular and extracellular recording techniques for studying circuits and reflexes, changes in

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synaptic efficacy, and regeneration in terms of the individual nerve cells involved (see Kandel, 1976). For certain problems it would be an additional advantage if individual cells could be deleted one at a time and in their entirety, without damage to neighbouring cells. For example, in a situation where a single neurone evokes synaptic potentials in two post-synaptic cells that are electrically coupled, it is not possible by simple physiological tests to ascertain whether synapses are made onto one or both of the cells. Inevitably synaptic potentials occur in both of the cell bodies. While electronmicroscopy could be used to search for the sites of the anatomical contacts, it would be simpler to remove one of the postsynaptic neurones and determine if synaptic potentials still occur in the others. Similarly, removal of a cell from the C.N.S. of an animal might provide clues about its function and about the ability of other cells to compensate for the deficit.

In certain animals, genetic techniques have been used to eliminate individual cells or classes of cells, but this is only practicable if the generation time is short (see Harris, Stark & Walker, 1976); moreover the full extent of the lesion may be difficult to ascertain. At first it might appear that a simple solution would be to destroy the cell body of a neurone mechanically or with a laser (Fork, 1971). In many invertebrates, however, this does not lead to death of the remaining process of the neurone. In crayfish, lobster, and leech it is clear that processes severed from the cell body not only survive for periods of weeks or months, but can secrete transmitter, carry sensory impulses, form synaptic connexions, and fire rhythmically (Hoy, Bittner & Kennedy, 1967; Nordlander & Singer, 1972; Carbonetto & Muller, 1977; Wallace, Adal & Nicholls, 1977; Van Essen & Jansen, 1977).

The present series of experiments show that the intracellular injection of the proteolytic enzyme mixture, Pronase, provides an effective method for killing cells in their entirety. This technique has been used (1) to show that one sensory neurone makes synaptic connexions with each of two motoneurones that are electrically coupled to each other, (2) to examine the functional role of a motoneurone, the deficit produced by its removal and the extent of recovery after other neurones take up its function. A brief description of the method has been presented elsewhere (Parnas & Bowling, 1977).

METHODS

The experiments were made on *Hirudo medicinalis*; animals were kept in 10% leech Ringer at room temperature. Techniques for recording from neurones with intracellular and extracellular electrodes and for injecting horseradish peroxidase have been described elsewhere (Muller & McMahan, 1976; Yau, 1976). Isolated ganglia were bathed in leech Ringer fluid containing (mM) NaCl, 115; KCl, 4; $CaCl_2$, 1·8; Tris maleate neutralized to pH 7·4 with NaOH, 10; glucose, 11·3. For culture, ganglia were maintained in L-15 medium with 5% fetal calf serum (see Wallace *et al.* 1977).

Pronase injection

Conventional micro-electrodes containing fibreglass were back-filled with Pronase (0·5%) in 50 mM-KCl. Fast green (2–4%) was added to provide visible confirmation of injection into a cell. Immediately before use the electrode was bevelled on a Schmeggegge beveller to a tip diameter of 1–2 μm and a resistance of about 100 MΩ. The procedure for injection with pressure was similar to that described for HRP. Usually a pressure of 1–5 lb./sq.in was sufficient when maintained for 1–5 sec.

Injections into anaesthetized animals

Animals were anaesthetized with 8% alcohol or 0·15% chlorobutanol and a small incision was made through the skin to expose the ganglion. The ganglion was illuminated at a shallow angle (from a fibre optic illuminator). This allowed cells to be recognized by their characteristic shapes, sizes, and positions; and after impalement the identification was confirmed by the characteristic action potentials. Animals lived for prolonged periods after the operation and ganglia could retain a normal appearance after the deletion of the cell. Frequently, however, because of adhesions to the blood vessel around the ganglion and failure of the blood vessel to heal, the operated ganglion became opaque. Similar changes have been observed after operations in which ganglia are exposed for prolonged periods (Jansen *et al.* 1974).

RESULTS

Destruction of neuronal cell bodies and processes by Pronase injection

Effects on cell body and axons

There was no immediate effect of Pronase on the electrical properties of leech neurones and, as with injections of HRP, the resting and action potentials were still present at the time the electrode was withdrawn. Over the next few hours, resting and action potentials progressively declined to zero. After a successful injection the fast green remained in the cell for 10–15 min; when the colour faded more rapidly, the processes of the cell were not killed. After about 12 hr the injected cells failed to exclude trypan blue and by about 2 weeks in the intact animal or in cultured ganglia the cell body could no longer be discerned. Pl. 1 shows ganglia in which one of the two large Retzius cells had been injected with Pronase. In *A*, 24 hr after the injection, the Retzius cell is stained with trypan blue while other neurones have excluded the dye. In *B*, a ganglion in organ culture that was injected a week earlier contains only a single Retzius cell.

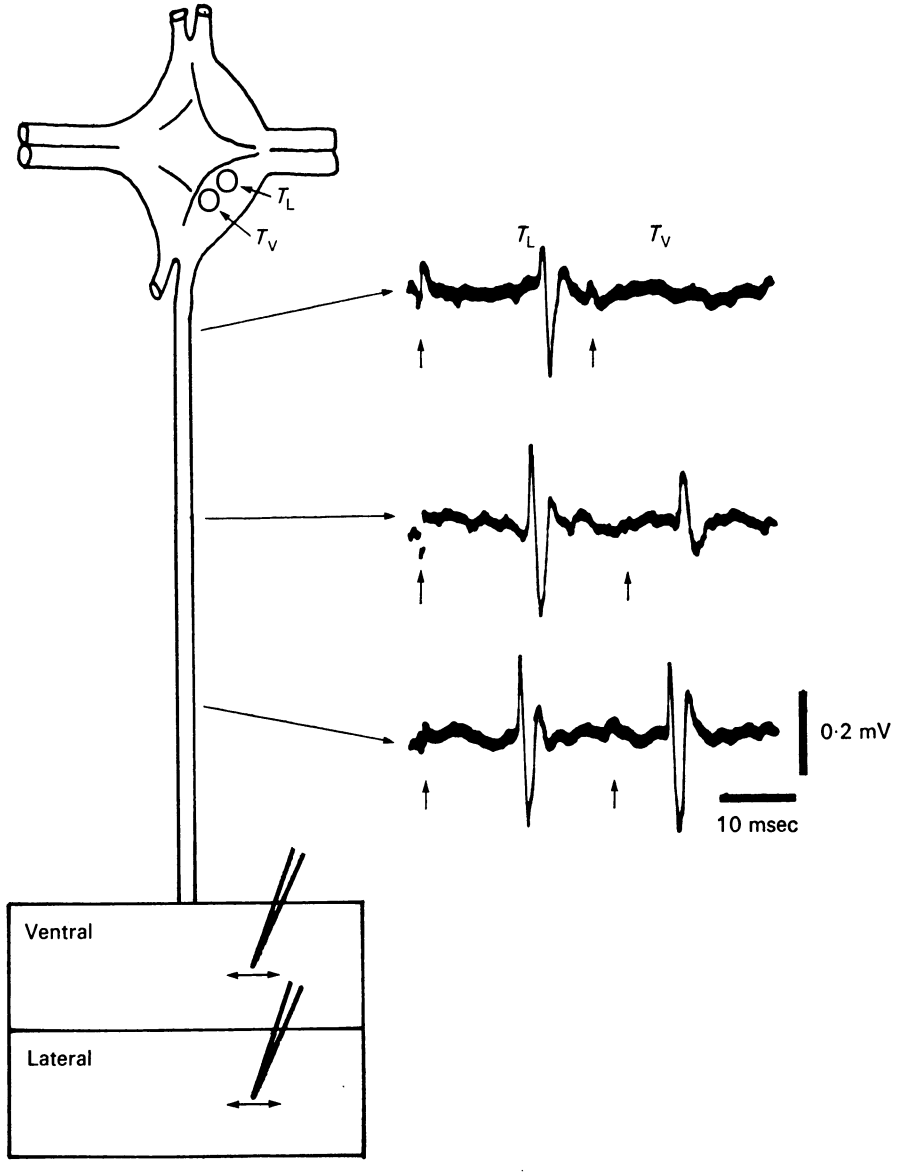
In control experiments, cells were injected with fast green and 50 mM-KCl or 50 mM-$CoCl_2$ by means of similar electrodes. Such cells could be recorded from hours or days after the injections and appeared to be completely normal.

Failure of conduction along distal processes

To ascertain whether Pronase killed the entire arborization of a neurone, Pronase was injected into touch-sensory neurones (T cells) which send long axons to their receptive fields in the skin. When the skin is touched, the T cell's impulses can be recorded in the root as they travel along the axon toward the cell body. These impulses persist for long periods after the cell body has been destroyed or cut off (Van Essen & Jansen, 1977). In the experiment in Text-fig. 1 the cell, T_L, innervated lateral skin; the other, T_V, ventral skin. The T_V cell body was injected with Pronase; 2 hr later its resting potential was absent and impulses could no longer be recorded intracellularly; but when the ventral area of skin was touched impulses could still be observed in the root with external electrodes. About 20 hr later, action potentials no longer spread as far along the injected cell's axon, while conduction along the uninjected axon remained normal. The time taken for failure of propagation to occur over the whole length of the injected cell's axon (approximately 4 mm) was about 24 hr. Similar experiments were made by injecting a single T cell in an intact animal

which was allowed to recover. After a week had elapsed, only two T cells (instead of three) could be found by intracellular recording. In addition, extracellular recording from the anterior root showed only one T cell response to stroking the skin and not two as in normal leeches.

These experiments did not reveal whether Pronase destroyed distal processes or



Text-fig. 1. Impulses recorded extracellularly from the axons of two sensory cells innervating lateral (T_L) and ventral skin (T_V). One cell T_V had been injected with Pronase 18 hr beforehand when its receptive field and that of T_L were mapped. At the time of the experiment, impulses initiated by touching ventral skin failed to propagate into the ganglion while those from lateral skin continued to do so. The arrows under the records indicate the instant at which the skin was indented by a piezo-electric crystal.

whether it simply blocked conduction. To test for destruction of axons as well as cell bodies, Pronase was injected into a small neurone that lies close to the Retzius cell, the S cell. Its axon is large and distinctive, and can be readily identified in the medial connective ('Faivre's nerve', Pl. 2*A*) by light microscopy (see Bagnoli, Brunelli & Magni, 1972; Frank, Jansen & Rinvik, 1975). Plate 2*B* shows a cross section of a preparation in which the S cell had been injected 2 days beforehand and then maintained in organ culture. The axon of the S cell, which is apparent in the control section (Pl. 2*A*), has collapsed, and cannot be recognized (see also Pl. 4).

A second method for demonstrating spread of Pronase through processes within the neuropile is shown in Pl. 2*C*. In this experiment two touch cells were injected with horseradish peroxidase and then left in culture medium for several hours to allow spread. One of the cells was then injected with Pronase, while the other cell was injected with KCl and fast green (see Methods), and the ganglion again left in culture to allow spread. The T cell in the upper portion of Pl. 2*C* is the control, showing a normal arborization within the neuropile. The T cell injected with Pronase (Pl. 2*C*, lower part of ganglion) contains only occasional deposits of the reaction product, without a clear outline of the cell body or its processes. At a greater distance from the cell body, however, the stained axons of the HRP-Pronase injected T cell could be clearly distinguished and appeared normal. Hence, the absence of reaction product within the neuropile cannot be attributed to an unsuccessful initial injection of HRP.

Synaptic connexions and reflex pathways

Monosynaptic connexions between sensory and motoneurones

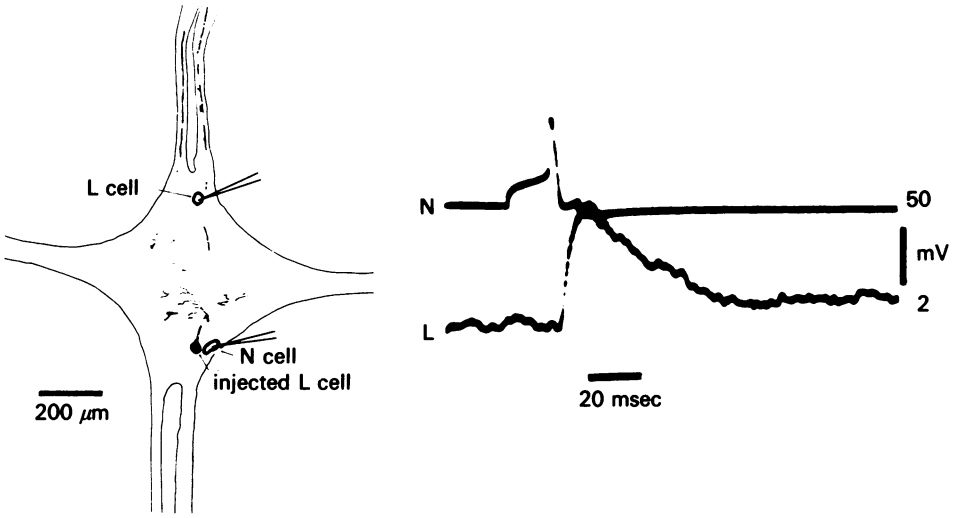
Physiological experiments have shown that sensory neurones responding to pressure (P), and noxious (N) mechanical stimulation evoke monosynaptic potentials on the L motoneurones, which are electrically coupled (Nicholls & Purves, 1972). N cells act by chemical synapses and P cells act by a combination of chemical and electrical mechanisms. The following experiments show that N and P cells are connected directly to both of the L cells in a ganglion.

Text-fig. 2 shows an experiment in which one L cell was injected with Pronase and the ganglion left in culture medium overnight. When intracellular recordings were made the next day no resting or action potentials could be recorded from the injected L motoneurone. In contrast, the other L cell appeared normal and synaptic potentials were evoked in it by stimulation of N and P cells on both sides of the ganglion. The records in Text-fig. 2 show an excitatory potential similar to that seen in control ganglia following stimulation of the contralateral N cell. To check that the L cell was destroyed by Pronase, HRP had been injected first, followed by Pronase a day before recording electrically. The camera lucida drawing in Text-fig. 2 shows that only traces of HRP were present in the neurone within the ganglion, indicating that it had been destroyed.

Elimination of the AE motoneurone

Each of the bilateral annulus erector motoneurones (AE cells) raises the skin into ridges on the contralateral half of the leech's body wall (Stuart, 1970). To see whether other, unidentified cells in the ganglion might also produce annulus erection one AE

cell was deleted. A diagram of the field of innervation of the contralateral body wall supplied by the AE cell is shown in Text-fig. 3. The territory consists of the entire segment and in addition, two annuli of the segment in front and two of the segment behind. Since each segment contains only five annuli it follows that much of the territory is also innervated from neighbouring ganglia. Only the central annulus receives input from a single ganglion. Accordingly, one would expect that destruction of a single AE cell in a single ganglion should lead to abolition of annulus erection over the single central annulus; destruction of two AE cells in adjacent ganglia should involve six annuli. The experimental procedure was to anaesthetize leeches, expose

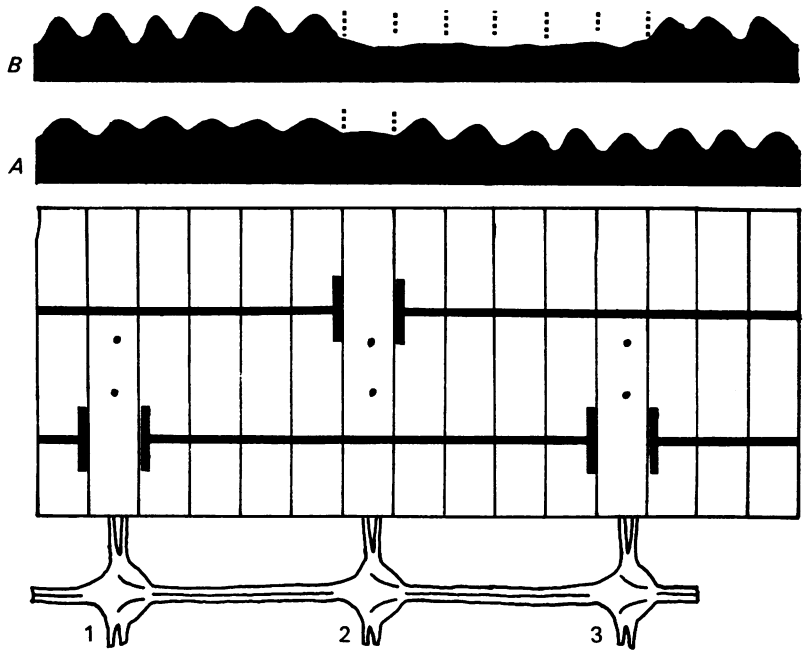


Text-fig. 2. Presynaptic action potential (N) and post-synaptic e.p.s.p. recorded from the contralateral motor cell (L). The ipsilateral L cell had been destroyed by an injection of Pronase 24 hr earlier. Camera lucida drawing of this L cell. 24 hr before the Pronase injection, HRP had been injected into the L cell. As a result only peripheral processes contain normal HRP deposits.

the nerve cord and inject one AE cell in either a single ganglion or in two adjacent ganglia. After recovery, the reflex was initiated by stroking the leech or blowing on it. The results are shown in Text-fig. 3*A* and *B*. As expected, after a single AE cell had been killed, the central annulus remained flat while the others became erect over the rest of the animal (Text-fig. 3*A*). When two AE cells had been killed (Text-fig. 3*B*) six annuli were flattened. From light and electron microscopic studies by Muller & McMahan (1976) it is known that the AE cell does not form presynaptic terminals on other neurones within the ganglion. These results therefore suggest that the AE cell alone mediates the motor command causing erection of annuli.

In animals kept for a long period after their AE cells had been killed, functional recovery of the annulus erection reflex progressed slowly over the paralysed region of skin. An example is shown in Text-fig. 4. In this animal, after six months, four of the six annuli that had originally been flattened became erect in response to sensory stimuli. The animal was dissected and experiments were carried out to identify the cells that mediated the recovered reflex. Text-fig. 4 is a drawing of the skin and

associated ganglia as they were pinned out. The two central ganglia are those in which AE cells innervating the right side of the animal had been killed with Pronase 6 months earlier. AE cells in the two non-injected ganglia were stimulated by intracellular current injection and their fields of innervation were mapped by noting the area over which the annuli became erect. Their fields are represented by the stippled areas in the Figure. On the right side of the animal the fields of the AE cells had expanded into the region formerly controlled by the killed cells. On the left side of the animal, which served as a control, the fields of the AE cells had normal boundaries.



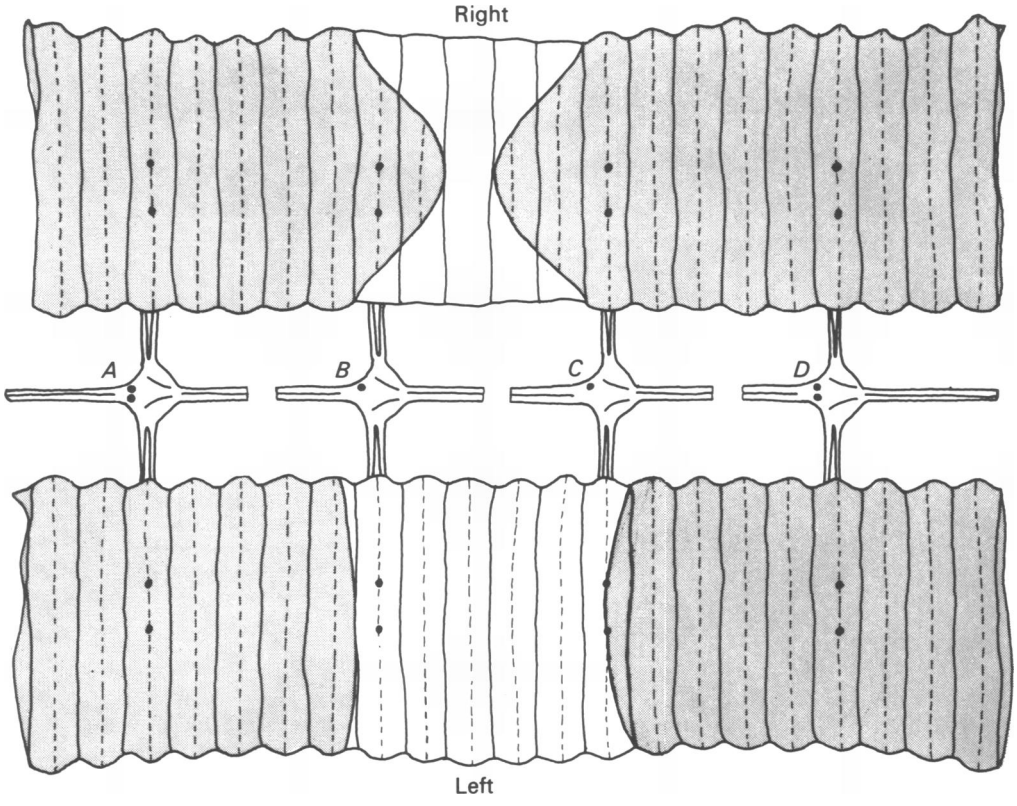
Text-fig. 3. Diagram (below) showing extent of normal innervation by the annulus erector cells. Each AE cell innervates muscles in the five annuli of its own segment and two additional annuli of the adjacent anterior and posterior segments. *A*, photographic silhouette of the edge of a leech illustrating the erection of its skin into ridges following stroking. One AE cell in ganglion 2 had been injected with Pronase 24 hr earlier. The central annulus remained flat while the other annuli of the segment innervated by AE cells in 1 and 3 became erect. *B*, photographic silhouette of a leech after two AE cells in ganglia 2 and 3 had been injected with Pronase 24 hr earlier. Six annuli failed to erect.

Does Pronase injected into a cell damage other neurones in the ganglion?

To test for subtle forms of damage to uninjected cells one of the two large Retzius cells was injected with Pronase. The Retzius cells lie close to one another in the ganglion, are tightly coupled by electrical synapses, and have coextensive arborizations in the neuropile. The uninjected Retzius cell showed no electrophysiological or morphological signs of damage two weeks after injection in the whole animal. The resting potential, action potentials, and input resistance of such neurones remained normal. In addition, the arborization seen by HRP injection in the remaining

Retzius cell was normal in its extent (Pl. 3) and the fine processes did not show the beading or discontinuities characteristic of Pronase injected cells (Pl. 2).

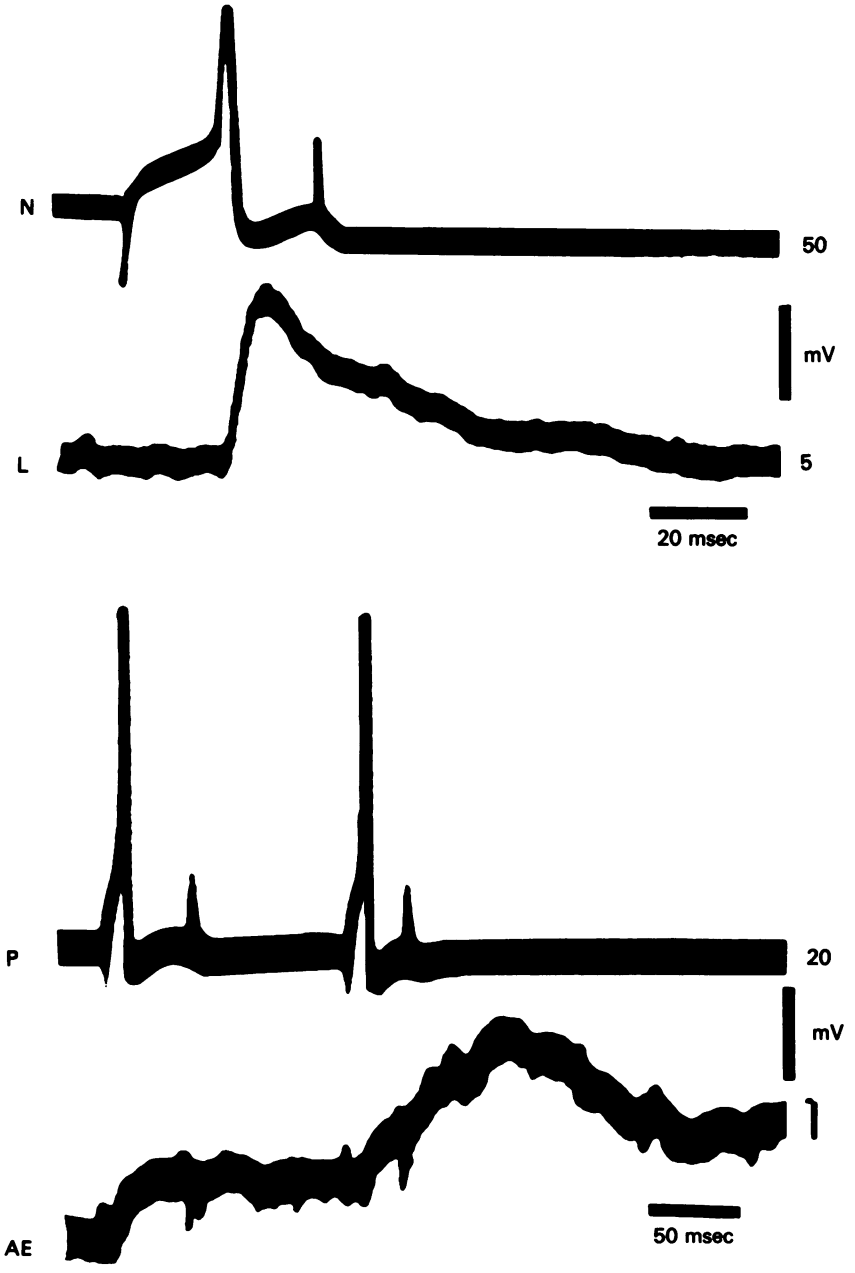
A second test for non-specific damage was to inject several cells (up to ten) in a ganglion. These included most of the T, P, and N cells as well as Retzius cells and a few unidentified neurones. The next day after maintaining the ganglion in culture the injected cells were dead, but the ganglion appeared otherwise normal. Uninjected cells retained normal properties and the remaining N and P cells still gave rise to characteristic synaptic potentials in the L and AE motoneurones (Text-fig. 5).



Text-fig. 4. Diagram of a dissected preparation from a leech in which AE cells (in ganglia *B* and *C*) innervating the right side had been injected with Pronase 6 months earlier. Immediately before performing the experiment, connectives were cut between ganglia *A*, *B*, *C* and *D*. Intracellular stimulation of the AE cells in ganglia *A* and *D* showed that those which innervated the right side of the animal had expanded their fields of influence into the region flattened by the earlier destruction of AE cells in ganglia *B* and *C*. On the left side where the remaining AE cells of ganglia *B* and *C* still innervated the skin, the cells of ganglia *A* and *D* did not expand their field size.

At the fine structural level no obvious changes were observed in uninjected neurones in electron micrographs of connectives after a cell had been killed. In one series of experiments in isolated pairs of ganglia the S cell which has a clearly identified large axon in the medial connective (see Pl. 2*A*) was injected. By 2 days in culture the S cell axon had disappeared, but the electron microscopic appearance of the remainder of the tissues shows no obvious signs of damage. Pl. 4 shows a repre-

sentative electron micrograph. In another series of experiments made independently by Dr K. J. Muller similar results were obtained with deletion of the S cell axon and little or no overt damage to other fibres. (personal communication).



Text-fig. 5. Synaptic interactions (N and P sensory cells to L and AE motoneurones) in a ganglion in which nine other neurones had been injected with Pronase 24 hr earlier. The synaptic potentials resemble those seen in normal ganglia.

DISCUSSION

The method described in this paper makes it possible to destroy a single neurone with little or no overt damage to others. The method by which Pronase spreads through the neurone is not clear. Failure to conduct impulses along axons in the root proceeded at a rate of about 0·2 mm/hour. This figure cannot, however, be simply correlated with diffusion or axoplasmic flow since the concentrations are unknown. Nor is it known how the neurone is destroyed by Pronase. In squid axons Pronase reduces sodium inactivation leading to a prolongation of the inward sodium current (Armstrong, Bezanilla & Rojas, 1973). In injected leech neurones the action potential failed but without an obvious increase in duration. Presumably Pronase spreads throughout all of the processes of the neurone, like horseradish peroxidase, and disrupts intracellular proteins with relatively little leakage to the outside. Even cells that are electrically coupled to an injected cell survive with no impairment in their electrical properties or disruption of their processes. However, physiological tests and electron microscopy do not rule out the possibility that Pronase leaks out of the injected cell and produces non-specific damage to glial or neuronal processes. One feature of Pronase action that would tend to limit such effects is that the enzymes in Pronase gradually lyse each other terminating their activity (Nomoto *et al.* 1960).

One application of the technique is to test whether an individual cell performs a particular function in the animal. Destruction of the AE cell caused the appropriate annuli in the leech to remain flat while those over the rest of the animal became erect. Since this AE motoneurone is not presynaptic to other neurones within the ganglion (Muller & McMahan, 1976), one can conclude that it is the only annulus erector motoneurone. After the AE cells had been killed, annuli remained flat for several weeks. Gradually the affected area became smaller through spread of activation from the cells in neighbouring ganglia. One possibility, for which there is no direct evidence, is that sprouting occurred and new neuromuscular synapses were formed. Similar slow spread of receptive fields is seen with sensory cells responding to touch after partial denervation of the skin; the fields of remaining touch cells increase in size only over periods of months (S. Miyazaki & J. G. Nicholls, unpublished) compared to the three weeks or so required for reinnervation of the skin or the annulus erector muscles after crushing the roots (Van Essen & Jansen, 1977).

The technique of Pronase injection also facilitates the analysis of synaptic connexions between sensory and motor cells. From electrical recordings one cannot infer whether each N sensory cell makes synapses on one or both L cells. The size and time course of the synaptic potentials are not adequate clues since the sites at which the neurones are coupled are not known. After Pronase injection, however, it was clear that individual N sensory cells make connexions onto both L motoneurones. To try to establish this by electron microscopy would be a formidable task.

Pronase injection may also be applicable to other problems concerning plasticity, sprouting, the role of glial cells and regeneration. Until now a serious difficulty in investigating long-term changes in synaptic efficacy or the specificity of regeneration has been the massive scale of lesions that are made during the experiment. From earlier work it is known that when neurones in the leech C.N.S. regenerate they accurately reform their connexions (Wallace *et al.* 1977; Carbonetto & Muller, 1977).

In addition, they form certain novel connexions. At the same time the lesion itself, destroying thousands of other fibres, produces alterations in synaptic organization, sprouting, and the appearance of synaptic interactions not seen in normal ganglia (Jansen *et al.* 1974; Wallace *et al.* 1977). With Pronase injection it should be possible to remove selected postsynaptic cells or selected presynaptic inputs and determine whether compensatory sprouting and rearrangement of synapses occur. Similarly, individual glial cells could be destroyed to observe how their chronic removal affects neuronal function.

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EXPLANATION OF PLATES

PLATE 1

Destruction of Retzius cells by intracellular injection of Pronase. *A* and *B* show two ganglia maintained in organ culture. One of the two Retzius cells in each of the ganglia had been injected with Pronase at the time of removal from the animal. In *A*, at 24 hr, the injected Retzius cell has become stained when the ganglion was bathed in Ringer fluid containing 0·2% trypan blue. No other cell was stained. This provides a rapid technique for demonstrating the death of a cell. In *B*, at 1 week, the injected cell body can no longer be discerned.

PLATE 2

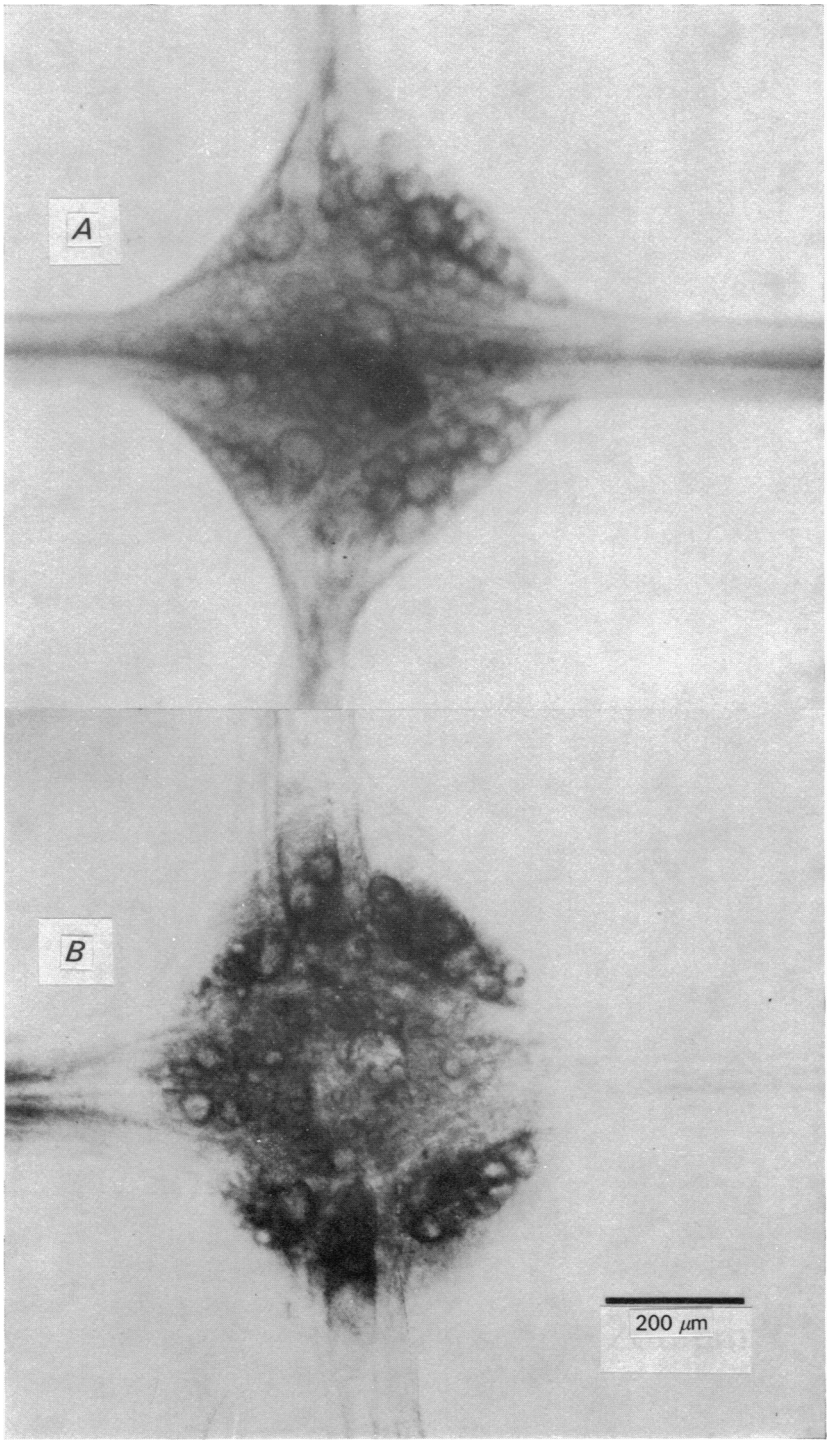
A and *B*, light micrographs of leech connectives in cross section. The control connective (*A*) shows a normal *S* cell axon in Faivre's nerve. In *B* is a connective 48 hr after the S cell body had been injected with Pronase with the result that the S axon was destroyed. In *C* is a leech ganglion in which one touch cell on each side of the ganglion was injected with HRP. Four hours later, the touch cell on the lower side was injected with Pronase. After another 15 hr, the peroxidase reaction was allowed to develop. The cell injected with Pronase showed only scattered processes within the neuropile (*E*) but its axons could still be discerned at a distance from the cell body in roots and connectives, indicating that the HRP injection had been successful. The processes of the other touch cell (*D*) appeared normal.

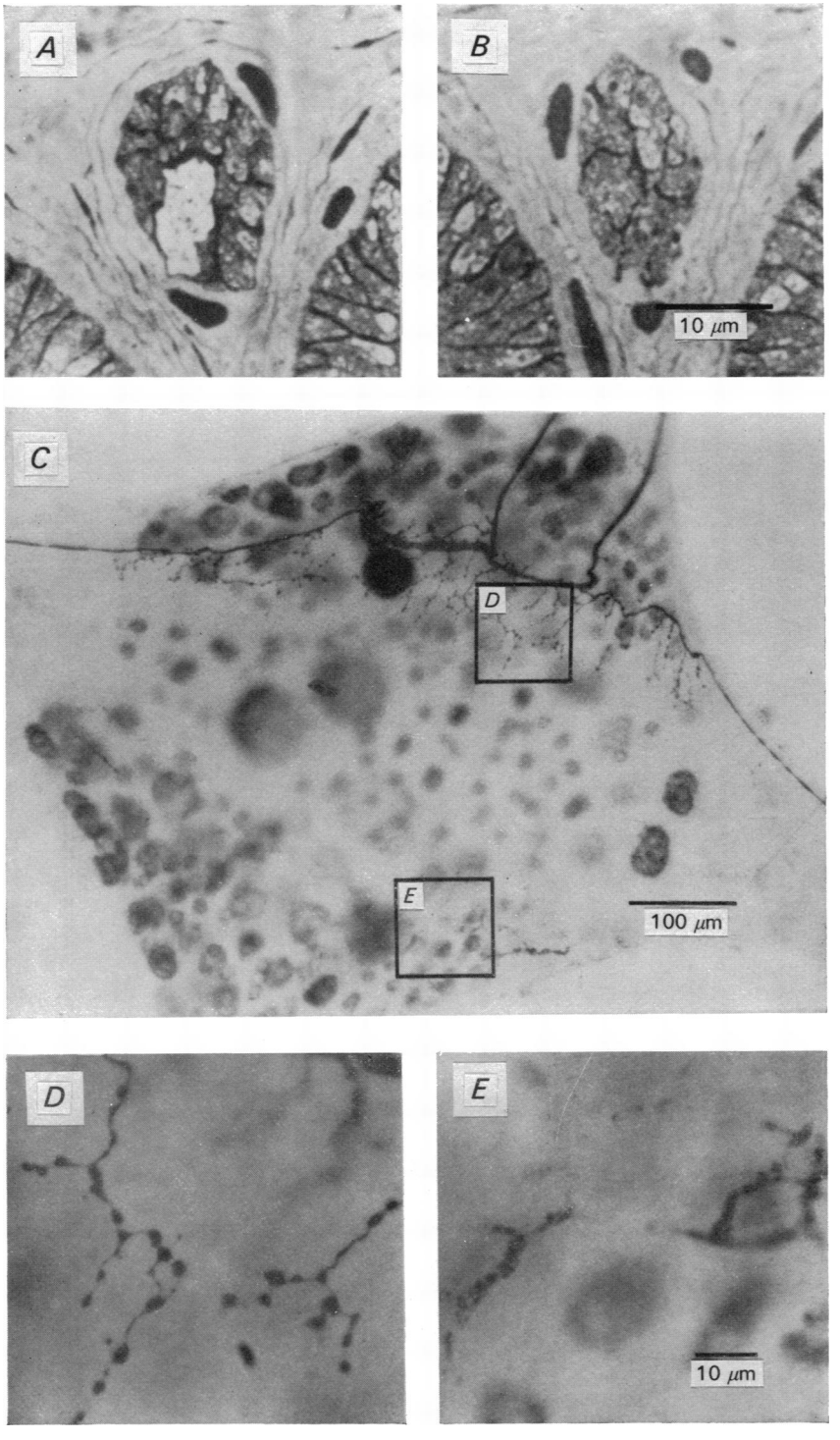
PLATE 3

Camera lucida drawing of Retzius cell injected with HRP and a photograph of some of its fine processes in the neuropile. Two weeks earlier, the other Retzius cell in the ganglion had been injected with Pronase. The Pronase injected Retzius cell had disappeared by this time but the arborization of the cell injected with peroxidase (which had been coupled to the Pronase injected cell) still appears normal. The stippled area represents the cell body.

PLATE 4

Electron micrograph showing a cross-section through Faivre's nerve 2 days after the S cell had been injected with Pronase. The S cell's axon which is normally 3–5 μm in diameter (Pl. 2*A*) is not present. Magnification, 8,500×.


A
B
200 μm


A
B
10 μm
C
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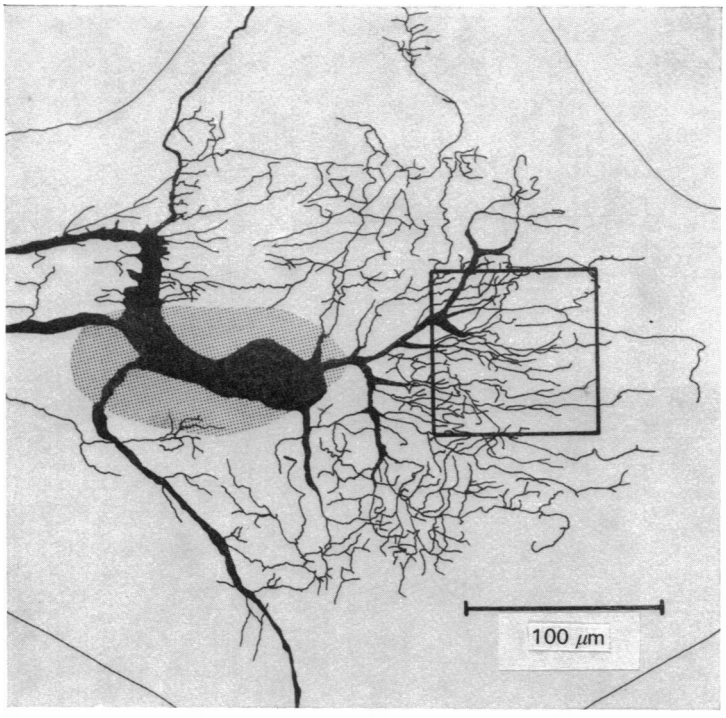
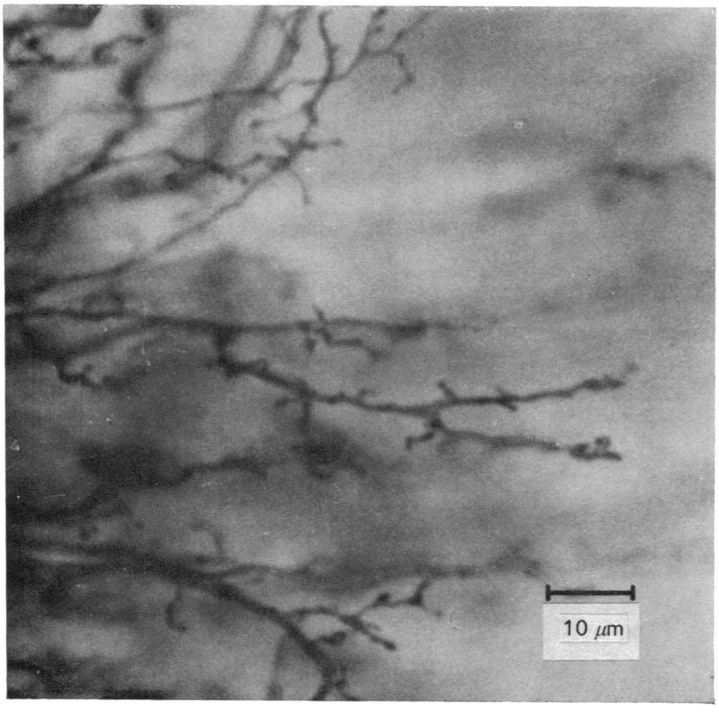
D
E
10 μm


100 μm


10 μm