Journal of Physiology (1991), **441**, *pp.* 733–754
With 7 figures
Printed in Great Britain

SYNAPTIC INTEGRATION AT A SENSORY–MOTOR REFLEX IN THE LEECH

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(*Received 16 May 1990*)

SUMMARY

1. In the medicinal leech the distribution of synapses from the pressure sensory (P) neurone to the annulus erector (AE) motoneurone and the site of impulse initiation in the AE cell were determined to understand better the integration of sensory inputs by the motoneurone.

2. The axon of the AE cell bifurcates before leaving the ganglion. Laser photoablation experiments indicated that the axon proximal to the bifurcation is inexcitable. Two techniques, laser photoablation and measurement of impulse timing, each located the site of impulse initiation at the bifurcation.

3. The medial P cell makes a monosynaptic connection with the AE cell, eliciting an excitatory postsynaptic potential (EPSP) of 1–3 mV amplitude recorded in the AE cell soma.

4. Intracellular injection of dyes into separate cells showed that P cell branches appear to contact AE cell branches both ipsilaterally and contralaterally. Laser photoablation of selected portions of the P and AE cells' axons revealed functional contacts on both sides.

5. The primary axon bifurcation of the AE cell is the site of integration of synaptic potentials that spread passively from both sides of the ganglion. These summed synaptic potentials account for the concerted activity of the two AE cells in each ganglion.

INTRODUCTION

One step toward understanding how sensory inputs lead to appropriate motor behaviour is to learn how individual motoneurones process sensory information. How do the diverse inputs to a single cell interact to determine whether the cell fires? The monosynaptic sensory–motor reflex is useful for understanding synaptic integration, in part because it has a limited number of elements and because it may elicit obvious behaviour. But even a two-cell reflex can involve multiple contacts from one cell that are spatially distributed on the other. Although the contacts may work in concert, little is known about the effectiveness of separate contacts that

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together produce a monosynaptic potential. In particular, do the different contacts driving a monosynaptic connection have different actions? Moreover, do inputs sum at a single integration site in neurones with no obvious axon hillock-initial segment?

To understand the efficacy of synaptic inputs it is useful to locate the synapses and the initiation zone in the postsynaptic neurone. Various approaches have been developed to this end. Synapses made by muscle sensory afferents on homonymous motoneurones in vertebrate spinal cord have been located by intracellular staining (Brown & Fyffe, 1981; Grantyn, Shapovalov & Shiriaev, 1984; Lichtman, Jhaveri & Frank, 1984), as have sensory afferent contacts in the leech central nervous system (CNS) (Macagno, Muller, Kristan, DeRiemer, Stewart & Granzow, 1981; Macagno, Muller & DeRiemer, 1985). Several techniques have been used to examine the distribution of *functional* contacts. For example, photoinactivation of a portion of the dendritic arbor of a dye-filled neurone has been used to study the wind-sensitive afferent input to separate groups of a cricket interneurone's dendrites (Jacobs, Miller & Murphey, 1986) and the functional contacts between two leech motoneurones (Lytton & Kristan, 1989). Another approach to study the distribution of functional contacts has been to use conduction block in conjunction with intracellular staining (Muller & Scott, 1981; Macagno, Muller & Pitman, 1987). The site of impulse initiation in most vertebrate neurones including motoneurones is at the axon hillock-initial segment, a morphologically distinct region of the axon near where it emerges from the soma (Eccles, 1964; Waxman & Quick, 1978; Kuffler, Nicholls & Martin, 1984), but in many invertebrate motoneurones and other neurones it is less clear where impulses originate. Focal extracellular and intracellular electrical recordings and optical recordings have been used in invertebrates, but the applicability of these techniques to locate the site of impulse initiation and synaptic integration has been limited.

Sensory–motor reflexes in the leech are particularly suited for examining the relationship between neuronal function and structure. Mechanosensory and motor neurones have been identified, and sensory receptive fields and muscle targets are well defined (Blackshaw, 1981*a*). Individual sensory neurones may make many separate synaptic contacts with a particular motor neurone (Macagno *et al.* 1987), but little is known concerning the distribution of inputs on the post-synaptic cell or concerning the convergence of inputs from several sources. It is not known precisely where in motoneurones the synaptic potentials sum to elicit an outgoing impulse, but recordings in the soma suggest that impulses arise at a distance from it.

The medial pressure (P) sensory neurone and the annulus erector (AE) motoneurone selected for this study are plainly visible on the ventral aspect of the leech ganglion and are accessible for laser microsurgery. The medial P cell innervates ipsilateral dorsal skin associated with its segment, projecting axons out along segmental nerves (roots) and the ipsilateral nerve cord (connective). The other element of the reflex, the AE cell, extends across the ganglion a large axon which bifurcates and leaves via the roots to innervate contralateral muscles that cause annuli surrounding the animal to form ridges. Although the two AE cells within each ganglion are weakly coupled through an electrical synapse, an impulse in one AE cell does not excite an impulse in the other, nor does dye pass perceptibly between the cells. The P cells are not coupled. From observations in which the P cells and the AE

cell have been stained separately by intracellular injection of horseradish peroxidase, it is known that the P cells make synapses at varicosities along secondary branches that emerge from their primary axons within the neuropile, and that the AE cells, which are postsynaptic, receive synapses chiefly upon fine spines that study the secondary branches that emerge from their primary axons as they cross the ganglion (Muller & McMahan, 1976). Experiments combining microsurgical, morphological and electrophysiological techniques were performed to examine the functional contacts between the P and AE neurones and to locate the site of impulse initiation within the AE cell.

In the vertebrate spinal cord and sympathetic ganglia, different neurones appear to report to a target through separate dendrites (Lichtman *et al.* 1984; Purves & Lichtman, 1985). This has also been thought to be true for some invertebrate neurones (Jacobs *et al.* 1986). In the present study in the leech it was determined that (1) synapses to a motoneurone from a single sensory neurone are distributed on widely separate dendrites, (2) the converging inputs are consistently integrated at an identified site and (3) different sensory neurones can share common dendritic regions on the target.

METHODS

Animals and preparation

Leeches (*Hirudo medicinalis*) were 1–1½ years old. Most were bred in our laboratory from stocks obtained from Ricarimpex in Audenge, France. They were maintained in artificial spring water (0·5 g solid Forty Fathoms per litre H_2O; Marine Enterprises, Towson, MD, USA) at 18 °C.

Anaesthesia was by cooling to 4 °C and nerve cord section below the cephalic ganglion. Midbody ganglia were dissected by making an incision along the ventral midline and were pinned ventral side up in silicone rubber-coated (Sylgard 184, Dow–Corning) polystyrene petri dishes (Falcon 1007) containing leech saline (Nicholls & Baylor, 1968) of the following composition (mM): NaCl, 116; KCl, 4; Tris maleate pH 7·2, 10; and $CaCl_2$, 1·8. For recording synaptic potentials the concentration of $CaCl_2$ was 7·5 mM, with equimolar replacement of Ca^{2+} for Na^+.

Electrophysiology and cell marking

Conventional techniques were used for recording, stimulation and intracellular dye injection (Appendix C in Muller, Nicholls & Stent, 1981). In brief, for intracellular recording, microelectrodes pulled from thin-walled glass were filled with 4 M-potassium acetate; resistances were 25–30 MΩ measured in physiological saline. A bridge circuit was used to pass current while measuring voltages. The brief, biphasic deflection before the synaptic potential in some high-gain recordings was a radiative coupling artifact reflecting the presynaptic impulse and current pulse. It was generated by the negative capacitance circuitry of the amplifiers and depended upon positioning of the amplifier inputs and the shielding between them. Recordings from somata of pre- and postsynaptic cells were filtered at 2·5 kHz, digitized at 8 kHz with a Tecmar A/D converter, processed and stored in an IBM XT-compatible computer. Suction electrodes were attached to segmental nerves for extracellular recording and stimulation. Extracellular signals were amplified with a Grass P-15 preamplifier before A/D conversion. In experiments to measure differences in time of peak of impulse arrival, signals were sampled at 25 kHz. Unless otherwise mentioned, errors in measurements of time and voltage are ± S.E.M.

After neurones were studied electrophysiologically, they were pressure-injected with markers to study their morphology (Muller *et al.* 1981). Three different markers were used: horseradish peroxidase (HRP, Sigma type VI), Lucifer Yellow CH (LY, Sigma) and 5,6-carboxyfluorescein (CF, Kodak). Microinjection pipettes were filled with 20 mg/ml HRP in 0·2 M-KCl containing 2 mg Fast Green FCF (Fisher) per millilitre, or 50 mg/ml LY in H_2O, or 0·1 M-CF, pH 7; before use they

were bevelled to resistances of 70–100 MΩ measured in physiological saline (Yau, 1976). Fluorescent cells filled with LY or CF were viewed in live tissue using a Zeiss WL compound microscope equipped with a 100 W DC mercury lamp and epifluorescence optics (Zeiss filter set 487709). HRP-filled cells were fixed, reacted with diaminobenzidine and H_2O_2, and mounted whole (Muller & McMahan, 1976; Muller & Carbonetto, 1979). Apparent contacts between neurones were detected by injecting LY into one and HRP into the other. Then ganglia were reacted before fixation in 4% paraformaldehyde for 30 min (Macagno *et al.* 1981). Cells were viewed using the following objective lenses: 10× (0·3 n.a. (numerical aperture)), 40× (0·75 n.a. water immersion), 40× (1·0 n.a. oil immersion), and 100× (1·3 n.a. oil immersion) with appropriate condensers.

The medial and lateral pressure sensory neurones on each side of a leech ganglion (Fig. 1*A*) innervate overlapping quadrants of ipsilateral dorsal and ventral skin (Nicholls & Baylor, 1968; Blackshaw, 1981*b*; Blackshaw, Nicholls & Parnas, 1982). P cells have been previously reported to produce monosynaptic excitatory postsynaptic potentials (EPSPs) in the AE cell, but no distinction has been made between lateral and medial P cells (Jansen, Muller & Nicholls, 1974; Muller & Nicholls, 1974). In the present experiments, the medial P (mP) cell was studied, because this cell, but not its lateral counterpart (lP), synapses directly with the AE cell (Fig. 1*B* and *C*).

Laser microsurgery

A laser microbeam was used to cut selected branches of the P and AE cells in the manner that we have previously used to axotomize touch (T) sensory neurones (Gu, Macagno & Muller, 1989). The beam was generated in a 35 mW argon laser (Lexel Corp., Palo Alto, CA, USA) and focussed through a Zeiss WL epifluorescence microscope (design of Dr J. Braun, see Gimlich & Braun, 1985). The laser was operated at 15 mW and was attenuated about 10-fold with neutral density filters. It was focussed through a Zeiss 40× water immersion objective lens (0·75 n.a.) to a spot approximately 5 μm in diameter.

Cells to be axotomized were injected with CF dye 5–15 min before cutting. Cuts were achieved by exposures lasting 2–6 s. Sometimes the ganglion was bathed in a leech saline containing 7·5 mM-$CaCl_2$ in order to promote resealing at a distance from the irradiation site, such as at central bifurcations of the mP neurone. The mechanism of the laser axotomy is not known; it probably involves the formation of free radicals within the cell (Spikes & Livingston, 1969). Axons adjacent to the cut axons but not filled with dye were unperturbed by the laser irradiation, as judged by their morphology when later filled with HRP (see also Gu *et al.* 1989). During irradiation there was little change in the dye intensity or its distribution, but within a few minutes the interruption in the axon became visible. The irradiated tissue was cultured in supplemented Leibovitz-15 (L-15) culture medium (Ready & Nicholls, 1979) for 2 h or longer to allow resealing and recovery of the irradiated cells before further study. At the conclusion of experiments, axotomized cells were injected with HRP and stained for morphological study.

RESULTS

Annulus erector motoneurone and its action potentials

The somata of AE cells, like those of other motoneurones in the leech and many other invertebrates, do not support action potentials (Stuart, 1970; Pitman, Tweedle & Cohen, 1972; Jansen *et al.* 1974; Muller & Nicholls, 1974; Heitler & Goodman, 1978). Since the AE cells' action potentials can be recorded extracellularly from segmental nerves where they emerge from ganglia, the site of impulse initiation is believed to be at a distance from the cell soma but within the ganglion. Impulses recorded in the soma, which is in a posteromedial location on the ventral surface of the ganglion (Figs 1 and 2), are normally only a few millivolts in amplitude (Fig. 2).

The AE cell has a structure typical of several other known leech motoneurones, as shown for an HRP-injected cell in Fig. 2*A*. The cell has a thick primary axon that crosses the ganglion and bifurcates (indicated by an arrow in Fig. 2*A*) before

projecting into the roots. Secondary branches emerging from the primary axon can be grouped as ipsilateral (represented by i in Fig. 2*A*) and contralateral (represented by c in Fig. 2*A*).

Excitable and inexcitable regions in AE cells

Where in the AE neurone are the impulses capable of being generated and where are they usually initiated? Is there a single origin, presumably the site at which

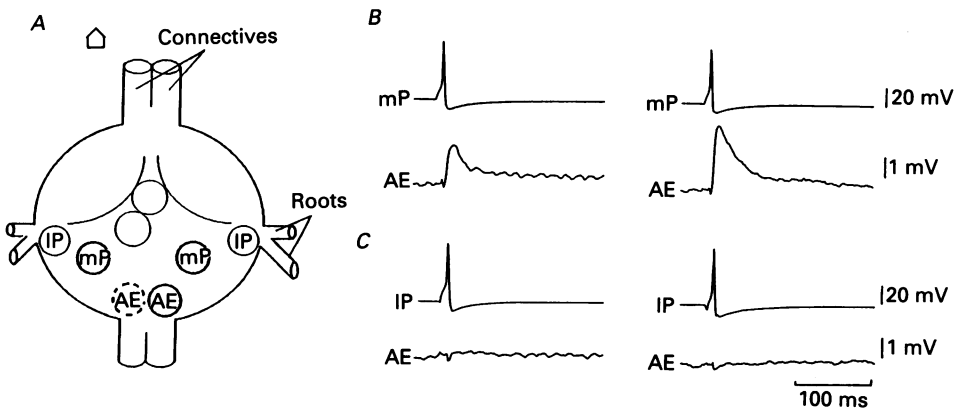

Fig. 1. Synaptic transmission from pressure sensory neurones (P) to the annulus erector (AE) motoneurone in a segmental ganglion. *A*, relative locations on the ventral surface of the ganglion of the four P cells – a pair of medial P cells (mP) and a pair of lateral P (lP) cells – and the pair of AE motoneurones (one AE cell with dashed outline, as in Fig. 6, was arbitrarily not used for physiological recording). The two cells in the middle of the ganglion, the Retzius cells, are unlabelled and are the largest in the ganglion. In this and the following figures anterior is toward the top (open arrow throughout). P cells were stimulated by passing depolarizing pulses of current through the recording microelectrode using a bridge circuit. Both mP cells strongly excite the AE cell (*B*), while the lP cell had little effect upon the AE (*C*). Left column shows recordings from the AE cell while stimulating P cells contralateral to it, while the right column shows recordings made while stimulating ipsilateral P cells. EPSPs were recorded in the AE cell when either mP cell was firing but not when the lPs fired.

synaptic inputs sum? To address these questions, the AE cells were axotomized at various locations. If the equivalent of an axon hillock-initial segment were removed, one might expect electrical excitability to be eliminated or greatly reduced.

AE cells were filled with CF dye and the laser microbeam aimed at their axons to sever them at particular sites. A critical point of this experiment was whether the usual small action potential remained after laser axotomy. Its disappearance in a well-resealed cell indicated that the remaining portion of the lesioned AE cell was inexcitable; persistence of the action potential indicated that the lesioned cell still included sufficient excitable membrane. More than 100 cells were irradiated and results were consistent. After two root axons were cut distally to the principal

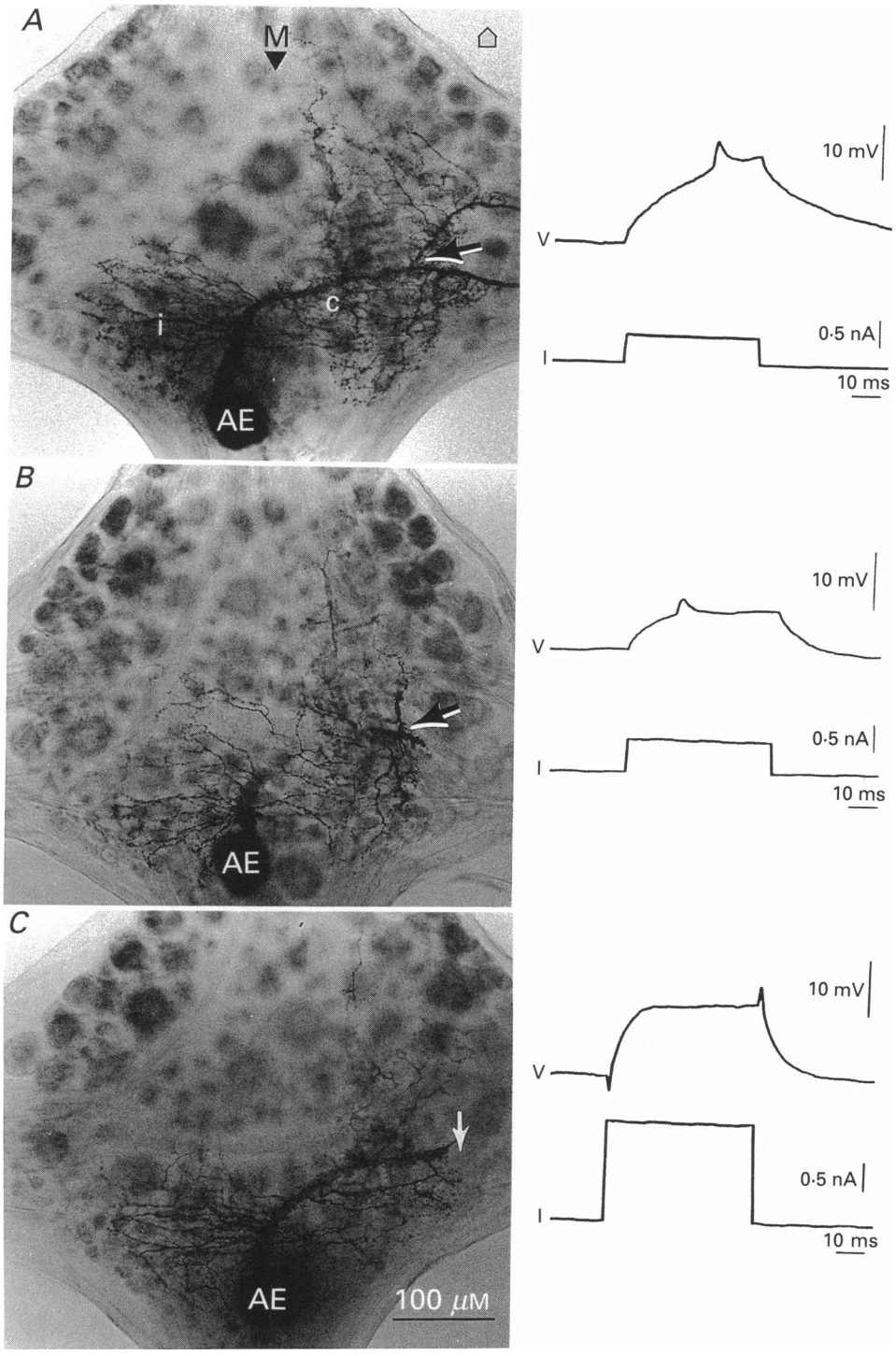

Fig. 2. For legend see facing page.

bifurcation within the ganglion, the usual small impulse could always be generated by depolarizing the soma through the recording microelectrode (Fig. 2*B*). Usually, resting potentials of AE cells are measured to be −40 to −55 mV. Following axotomy of twenty-nine cells distal to the primary bifurcation, resting potentials remained constant (±3 mV) in eleven and increased in twelve neurones. Input impedances in all ranged from 20 to 40 MΩ before cutting and from 25 to 55 MΩ afterward. As illustrated in Fig. 2*C*, when the axon was instead cut proximal to the bifurcation the impulse was invariably lost, whether or not input impedance rose above its normal level (Fig. 2*C*). In eleven cells measured, resting potentials remained the same ($n = 2$) or increased an average of 10 mV ($n = 9$). The white arrow in Fig. 2*C* indicates the original location of the lesioned AE cell's bifurcation. In contrast, excitability remained if the cell was irradiated proximal to the bifurcation, but without cutting the axon.

This result demonstrates that the membranes of the AE soma and axon proximal to the primary fork are inexcitable, therefore they are incapable of generating an action potential. The action potential originates only in the membrane of the root axons or the fork. Is the bifurcation the site at which impulses arise in response to synaptic inputs, or do impulses arise at one or more additional sites in the AE cell, such as in the root axon?

Site of impulse initiation in AE cells

To locate precisely where action potentials are initiated, and in particular to learn whether they arise at the axon bifurcation, the differences in times of arrival of action potentials were measured with pairs of electrodes as shown in Fig. 3*A*. Suction electrodes were attached to both the anterior (a) and posterior (d) roots, and a microelectrode was inserted into the AE cell body (c). Action potentials, generated spontaneously or by electrical stimulation, were recorded from both the soma and one root axon. The differences in time of arrival of the peaks of action potentials were determined.

The following analysis shows that if the spontaneous, synaptically driven impulse

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Fig. 2. Initiation of action potentials in normal AE cells and in AE cells axotomized at different locations with a laser. The left column of photomicrographs shows the morphology of intact (*A*) and laser lesioned (*B* and *C*) AE cells injected with HRP and stained after recording from them electrophysiologically, shown in corresponding traces in right hand column. In *A*, M at triangle indicates ganglion mid-line, while i and c represent ipsilateral and contralateral branches of the AE cell. In *A* and *B* a black arrow points to the AE axon's primary bifurcation, while in *C* a white arrow indicates the location of the AE cell axon's primary bifurcation before it was destroyed. In the right column, AE cells were stimulated with pulses of depolarizing current (*I*, lower traces) injected through the recording microelectrode into their somata. The upper trace (*V*) in each pair shows the corresponding voltage changes in the soma of the AE cell. Action potentials in the soma were less than 5 mV in amplitude, presumably because they were produced at a distance from the soma. Depolarizing pulses evoked action potentials in normal AE cells (top pair of traces) and in those AE cells in which the primary bifurcation remained after axotomy (middle traces), but they were absent after axotomy proximal to the bifurcation (bottom traces), no matter how strong the stimulus. Bridge artifacts are visible at the beginning and end of the strong pulse in bottom voltage trace.

arises at the bifurcation, the difference in arrival time at the soma and one suction electrode equals the difference measured for impulses generated with the other suction electrode. Moreover, the locations of other possible sites of impulse origin can be determined as described below.

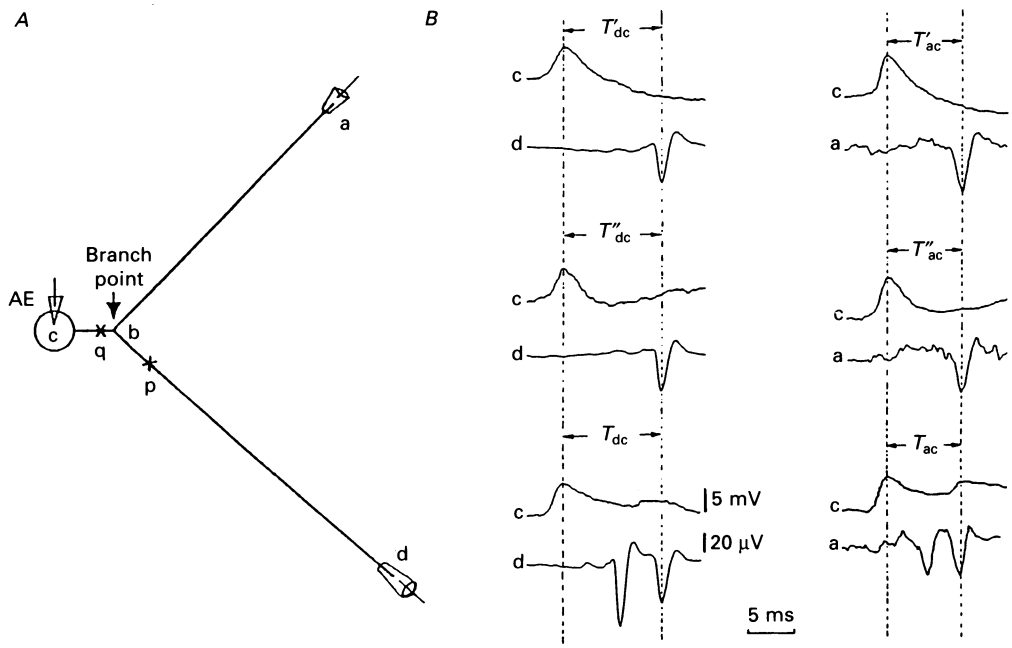

Fig. 3. Timing of action potential propagation can indicate the site of impulse initiation. Action potentials were initiated spontaneously or by stimulating the AE soma or one of the root axons, and the differences in time of arrival at different recording electrodes determined. The differences are used to determine where the action potential initiating zone is in the AE cell. *A*, the AE cell and the sites of recording electrodes a, c, and d, as well as the branch point, b. *B*, traces were recorded intracellularly from the cell body (c) and extracellularly from one or another root axon. The top pairs of traces were recordings of spontaneously arising impulses, those in the middle were evoked by stimulating the soma, and the bottom pairs were elicited by stimulating the anterior (a; left trace) or dorsal-posterior (d; right trace) root, respectively. When stimulating from roots, there is a peak of action potential appearing earlier than the peak being measured in both traces a and d that is associated with a cell(s) that conducts faster and has a lower threshold since it could be recorded alone by reducing the intensity of stimulation at the roots (not shown). A computer was used to measure each time difference (T'_{dc}, T''_{dc} and T_{dc}) between the peak of action potentials arriving at c and d for spontaneous, somatically evoked and root evoked action potentials, respectively. Measurements were made similarly in the right column, except that the three pairs of records were from c and a and time differences are represented with T'_{ac}, T''_{ac} and T_{ac}. Spontaneous impulses arose at the bifurcation or within 20 μm of it (see text). Sites p and q represent two hypothetical impulse initiation zones that can be distinguished operationally from b (see text).

When an action potential was generated by stimulating the anterior root axon (a), it propagated along this axon towards the bifurcation (b). From the bifurcation it propagated continuously through the posterior root axon and arrived at the suction electrode (d) applied to that axon. From the bifurcation it was also detected at the

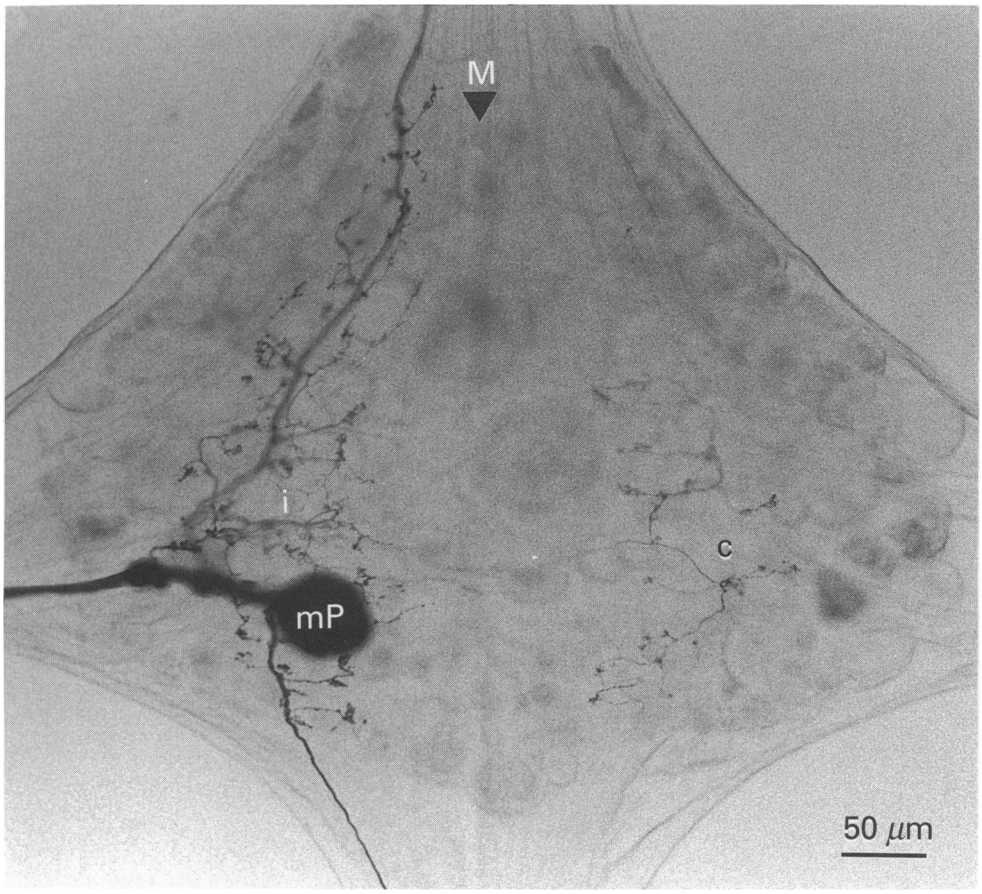

Fig. 4. Morphology of the mP cell. An mP cell was injected with HRP and stained. Round somata of other cells are visible in the background. The axons and most branches are ipsilateral (i), but a few branches are contralateral (c). M at triangle demarcates ganglion mid-line. Varicosities on both sides are sites of synapses (Macagno *et al.* 1987).

TABLE 1. Differences in action potential conduction delays for AE cells

Prep	T'_{ac}(ms)	T_{ac}(ms)	T'_{dc}(ms)	T_{dc}(ms)	ΔT_{ac}(ms)	ΔT_{dc}(ms)
1	5·19(±0·09)	5·23(±0·03)	8·08(±0·06)	8·00(±0·06)	−0·04	+0·08
2	10·27(±0·07)	10·34(±0·07)	7·78(±0·04)	7·92(±0·04)	−0·07	−0·14
3	2·07(±0·03)	2·12(±0·03)	3·20(±0·03)	3·22(±0·03)	−0·05	−0·02
4	9·32(±0·10)	9·55(±0·09)	5·24(±0·08)	5·22(±0·03)	−0·23	+0·02
5	5·55(±0·03)	5·47(±0·06)	8·77(±0·07)	8·65(±0·02)	+0·08	+0·12

Time differences between action potentials arriving at different electrodes (see Fig. 3*A* for configuration of electrodes and measurements). The data include measurements from five AE cells, listed as Prep 1–5. T'_{ac} is time between peak of spontaneously arising impulses recorded at the cell body (c) and anterior root electrode (a), while T_{ac} is the time between peak of impulses generated in the dorsal posterior root (d) and recorded at the same locations (a and c). Conversely T'_{dc} and T_{dc} are the time differences for the spontaneous and evoked impulses, respectively, recorded between the cell body (c) and dorsal-posterior root. Values are averages of more than ten measurements (for Prep 4, $n = 4$); errors are S.E.M. The $\Delta T = T' - T$ for the corresponding measurement. See text for conversion of ΔT into distance from the primary branch point.

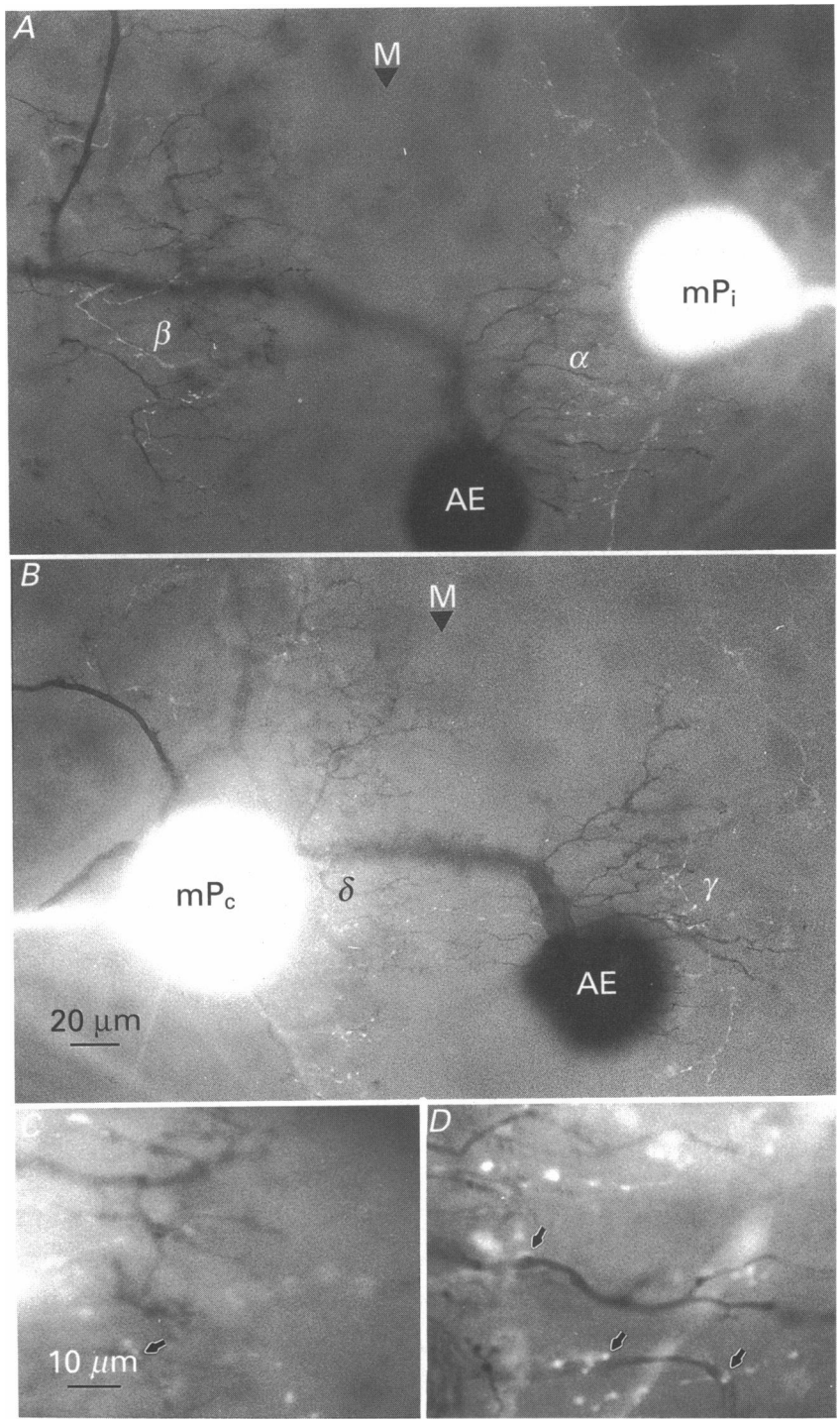

Fig. 5. For legend see facing page.

soma (c) through the main axon. The time between peaks of action potentials evoked at (a) arriving at the soma (c) and posterior suction electrode (d) was represented by T_{dc} (Fig. 3*B*); when instead the posterior root axon was stimulated, the interval between the soma (c) and the anterior electrode (a) action potentials was given as T_{ac}. For spontaneously arising action potentials the time differences between arrival at the soma (c) and at each suction electrode, (a) and (d), were designated as T'_{ac} and T'_{dc}. It was assumed that spontaneous action potentials and action potentials generated by EPSPs originated at one and the same impulse initiation zone, unless they were generated by electrical stimulation at or near a site of injury, such as the cut end of an axon.

If the impulse initiation zone were at the axon's principal bifurcation, one would expect $T'_{ac} = T_{ac}$ and $T'_{dc} = T_{dc}$. This is because for this site the action potential, whether initiated spontaneously or evoked by suction electrode (d), would travel from (b), the branch point, both to (a) in time T_{ab} and to (c) in time T_{bc}. Therefore we have $T'_{ac} = T_{ab} - T_{bc} = T_{ac}$. Similarly $T'_{dc} = T_{db} - T_{bc} = T_{dc}$, where T_{db} represents time for the action potential peak to travel from (b) to (d) and the anterior suction electrode is used for stimulation. This is true regardless of the conduction velocity in each axon branch. If instead the initiation zone were somewhere along one root axon, there should be a predictable departure from these equalities. If it is supposed, for example, that the initiation zone were at a point (p) along the *posterior* root (Fig. 3*A*), then $T'_{ac} = T_{ac}$, because action potentials initiated spontaneously or by suction electrode at (d) would go from (b) to both (a) and (c). However, one would expect $T_{dc} = T'_{dc} + 2T_{bp}$, where T_{bp} is the time required for an action potential to propagate from the initiation zone to the axon bifurcation. This is because when spontaneous action potentials are initiated at (p), $T'_{dc} = T_{pd} - T_{bp} - T_{bc}$. Since $T_{pd} = T_{bd} - T_{bp}$, $T'_{dc} = T_{bd} - T_{bc} - 2T_{bp}$, and thus $T_{dc} = T'_{dc} + 2T_{bp}$. To calculate the distance of p along the root from the branch point it is necessary to know the speed of conduction which can be approximated by measuring the distance from the soma to the root electrode and the conduction delay. There is no dependence on speed of conduction in the other root.

Laser transection experiments show that impulses reach the soma by a passive spread from an axonal initiation site at or near the bifurcation. If that were not true and the impulse were initiated proximal to the bifurcation, say at a point (q) (Fig. 3*A*), then there would be a positive delay T_{bq}, such that $T'_{ac} - T_{ac} = T_{bq} = T'_{dc} - T_{dc}$. If there were multiple or varying initiation zones, then in general, one would expect $T'_{ac} \neq T_{ac}$ and $T_{dc} \neq T'_{dc}$.

Experiments on several AE cells as illustrated in Fig. 3*B* showed that there was little difference between T'_{ac} and T_{ac}, or between T'_{dc} and T_{dc}, defined as ΔT_{ac} and ΔT_{dc}

Fig. 5. Distribution of branches of AE and both mP cells (mP_i, ipsilateral mP cell; mP_c, contralateral mP cell). Shown are regions of the neuropile in which branches of the AE motoneurone intermingle with branches of each mP cell in the ganglion. Double staining, with HRP in the motoneurone and LY in the mP cell, was used to show branches of the motoneurone and ipsilateral (*A*) or contralateral (*B*) sensory neurone simultaneously. *A*, two regions, ipsilateral (α) and contralateral (β) to the AE cell, are visible in which sensory and motoneurone branches appear to make contact at what may be synaptic sites. M indicates the ganglion mid-line. *B*, in the AE cell's ipsilateral (γ) and contralateral (δ) regions are apparent synaptic sites with the contralateral mP cell. Region δ is shown at higher magnification in *C* and region α in *D*, with apparent contacts indicated by arrows.

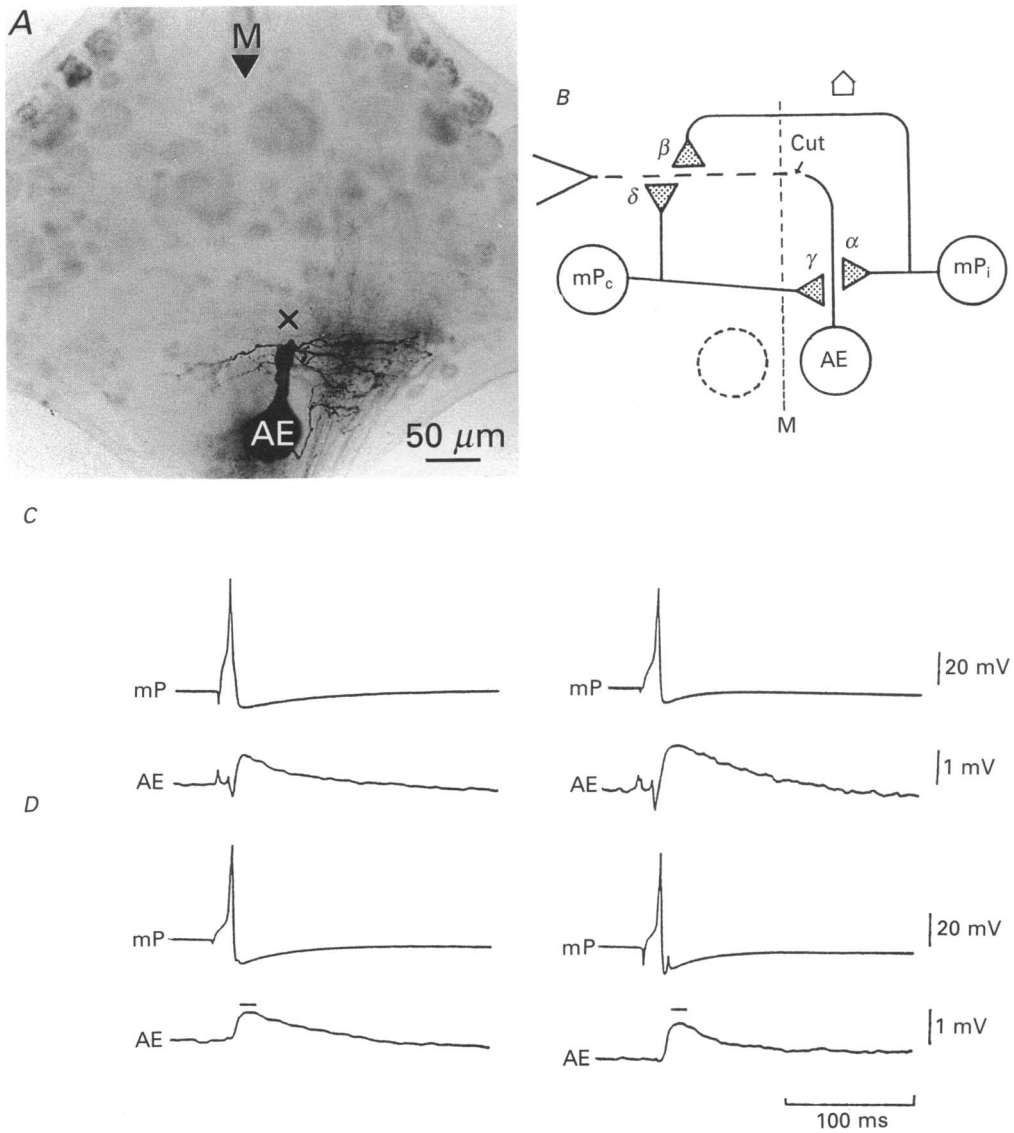

Fig. 6. Laser axotomy of AE cell alters transmission from two mP cells. *A*, the axon of an AE cell was filled with CF and cut by laser irradiation near the ganglion mid-line at the site indicated by a cross in *A*. The cell was later filled with HRP and stained, which revealed that the cell had lost its axon and all branches contralaterally, leaving only ipsilateral branches. M indicates ganglion mid-line. *B*, proposed regions for synaptic contacts between the AE and two mP cells, based on double-stained preparations having apparent contacts in regions in which sensory and motor neurone branches intermingle. Thus, each sensory neurone appears to make contacts (stippled triangles) both ipsilateral (α or γ) and contralateral (β or δ) to the AE motoneurone. These include the regions of possible contact between ipsilateral (α) or contralateral (γ) mP cells (mP_i and mP_c respectively) and the AE cell remaining after mid-lime axotomy of the AE cell. Open arrow indicates anterior. *C* and *D*, intracellular recordings from mP and AE cells before

respectively (Table 1). Measured time intervals from five preparations, listed in Table 1, show that the delays for spontaneous and evoked propagation were similar. By this criterion the site of impulse initiation was close to the principal bifurcation of the axon, if not at it. With the measured conduction velocity of 0·2 m/s in the two AE-cell root axons and assuming that conduction velocities in all parts of the two root axons are the same, a $\Delta T = 0{\cdot}2$ ms can be converted to distance: $\frac{1}{2}$ (0·2 ms) (0·2 m/s) = 20 μm, since ΔT is $2T_{bp}$ (see above). Thus, the scatter of the time measurements in Table 1 is equivalent to an uncertainty of about 20 μm or less for the site of initiation. Variation in conduction velocity would cause corresponding change in uncertainty, thus a 20% variation in conduction velocity would change the error by ± 4 μm.

Distribution of apparent contacts between mP and AE cells

The mP cell (Fig. 4) arborizes principally ipsilaterally within the ganglion neuropile, but also extends one or a few secondary branches across the ganglion to form a contralateral arborization. Enlargements along and at the ends of secondary branches are the sites at which the mP cell makes chemical synapses with other neurones and is itself postsynaptic (Muller & McMahan, 1976). Previous studies of the morphology of synaptic contacts between identified sensory and motor neurones in the leech have shown that apparent contacts seen in the light microscope using LY and HRP to stain separate neurones are reliably observed to be synapses when viewed using electron microscopy (Granzow, Friesen & Kristan, 1985; Macagno *et al.* 1987). We therefore used double staining to map the distribution of apparent contacts between LY-stained mP cells and HRP-injected AE cells (Fig. 5).

Low-power micrographs of doubly injected preparations in Fig. 5*A* and *B* illustrate the extensive region of possible contact in which the projections of the cells overlap. Observation at up to 1000× (Fig. 5*C* and *D*) showed apparent contacts both ipsilaterally and contralaterally of mP cells with AE cells on both sides of the ganglion. For simplicity in the present paper the ipsilateral contacts from each mP cell shall be considered as two units (α and γ) as shall the contralateral contacts (β and δ), as illustrated in Figs 6 and 7. Thus, double staining indicates that input from either the left or right mP cell may be received by the AE cell upon two different groups of branches.

(*C*) and after (*D*) axotomy. P cells were stimulated by pulses of depolarizing current passed through the intracellular microelectrode. Left column shows responses from the AE cell when the contralateral mP was stimulated by intracellular current injection and the right column shows responses when the ipsilateral mP was similarly stimulated. Transient depolarizing and hyperpolarizing deflections in the AE records before cutting are coupling artifacts produced by the depolarizing pulse in the sensory neurone electrode and the negative capacitance of the amplifiers; these were also visible after withdrawing the electrode from the cell. Electrophysiology was done before cells were filled with the markers HRP or LY. Axotomy reduced the amplitudes of the EPSPs from levels shown with horizontal bars.

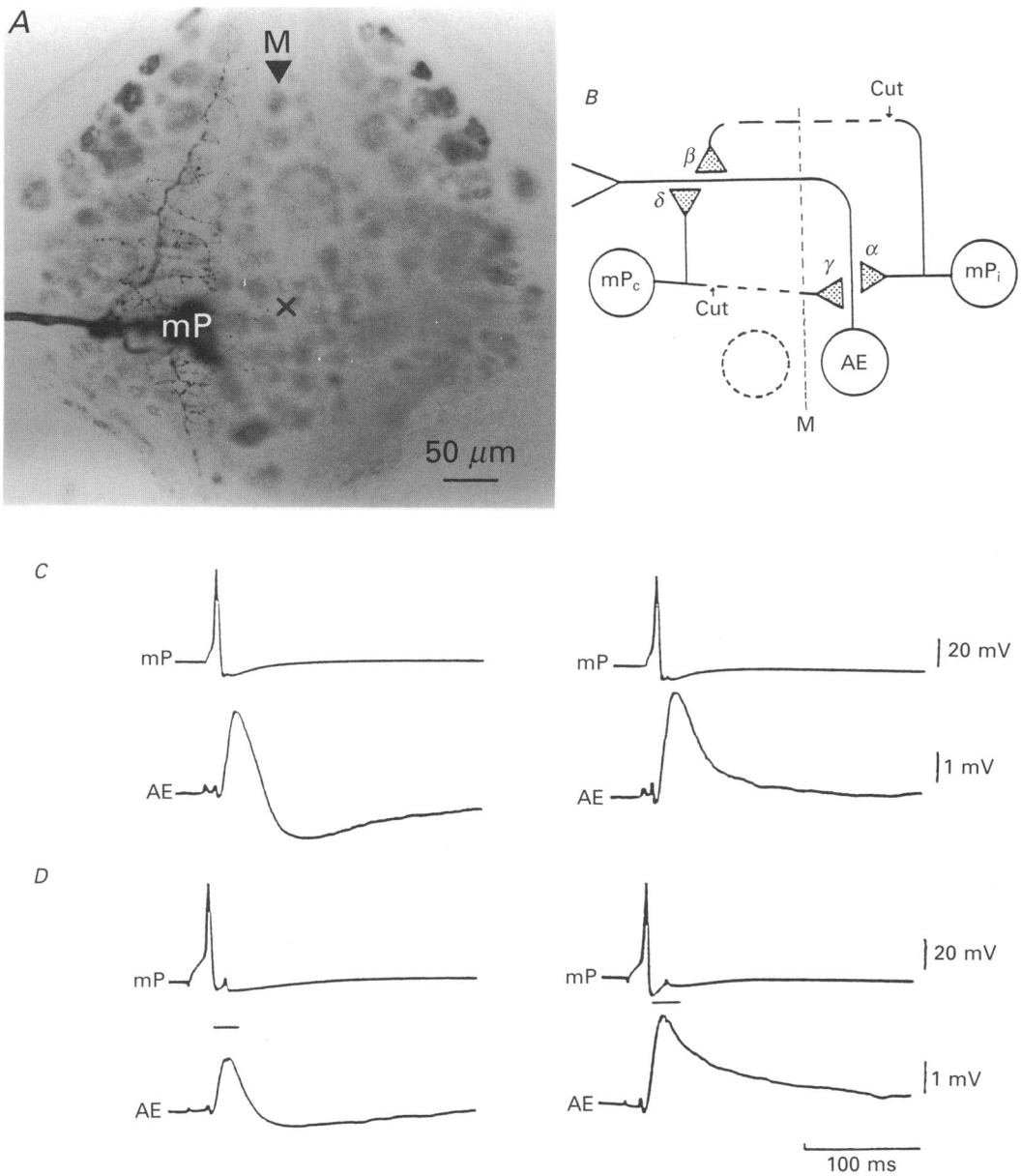

Fig. 7. Laser axotomy of mP cells alters transmission to AE motoneurone. *A*, contralateral branches of an mP cell were cut after filling it with CF and irradiating it with a laser microbeam at the site indicated by cross in photomicrograph. After cutting, the mP cell was filled with HRP and stained. Only ipsilateral mP branches remained. M marks ganglion mid-line. *B*, distribution of contacts with AE cell after cutting single mP cells' contralateral branches ipsilateral and contralateral to the AE. For experimental convenience, instead of cutting the contralateral branches of both mP cells in a ganglion, only one mP cell was cut. Transmission mediated by ipsilateral branches of the cut mP cell both to ipsilateral and contralateral AE cells in the ganglion was studied. *C* and *D*, intracellular recordings from the mP and AE cells before (*C*) and after (*D*) axotomy. As in Fig. 5, the P cell was stimulated by injecting current intracellularly. Left column shows

Laser axotomy reveals synaptic sites

Microbeam cuts 5,6-carboxyfluorescein-filled cells

To confirm that regions of apparent contact between cells were sites of functional synapses, portions of CF-filled cells were selectively removed by cutting with the microbeam of an argon laser and testing transmission between the remaining arbors. Lesions were made in saline containing 1·8 mM-Ca^{2+}, and recovery occurred during two or more hours in modified L-15 medium. The technique requires that transmission persists between those portions remaining, a condition that experiments showed was fulfilled.

Motoneurone axotomy

After the principal axon of the AE cell was cut within the neuropile, transmission could only have been to its ipsilateral branches (Fig. 6*B*). Cells selected for study had recovered their input impedance in the hours following axotomy and were therefore deemed to be healthy. It was found consistently after cutting that ipsilateral (all twenty-six preparations) and most contralateral (twenty-one of twenty-six preparations) mP cells continued to produce synaptic potentials of 1–2 ms latency in the AE motoneurone (Fig. 6*D*). Those five of twenty-six contralateral mP cells failing to produce synaptic potentials in the axotomized AE cell may have been mP cells that had no contralateral projection. The results are taken as direct evidence that the contact area α and, when it exists, contact area γ are sites of functional synapses between the ipsilateral and contralateral mP cells and the AE cells.

Comparison of EPSPs recorded in AE cells before and after axotomy showed that in most cases they became smaller, including in those AE cells whose input resistances increased after cutting. Fifty-two pairs of AE and mP cells were examined, twenty-six ipsilateral and twenty-six contralateral, with an average synaptic potential of $2{\cdot}97 \pm 0{\cdot}29$ mV ($3{\cdot}51 \pm 0{\cdot}32$ ipsilateral and $2{\cdot}42 \pm 0{\cdot}23$ contralateral). After axotomy the input resistance either increased or did not change in AE cells for forty-two sensory–motor pairs (twenty-one on each side) and for those the EPSP declined an average of $26 \pm 9\%$ ($20 \pm 10\%$ ipsilaterally and $36 \pm 9\%$ contralaterally). In contrast, in control experiments in which the two root axons of the AE cell were cut with the laser without removing contralateral branches within the neuropile, there was no change in synaptic potentials (not shown). This indicates indirectly that the contralateral branches of the AE cell at regions β and δ are also sites of functional synapses with the mP cells. In a few AE cells (four of twenty-six) the EPSPs slightly increased after cutting; a likely cause of this is discussed below.

Removal of mP cell contralateral branches

A direct test for contact at region δ (Fig. 6) was to sever the mP cell's contralateral branches rather then severing the axon of the AE cell. Contralateral branches were cut with a laser in twelve dye-filled mP cells as shown in Fig. 7, disconnecting the mP

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responses from the contralateral AE cell when the mP was stimulated and the right column shows the ipsilateral AE cell's responses when the mP was stimulated. Electrophysiology was done before cells were filled with the markers HRP or LY. Axotomy reduced the amplitudes of the EPSPs from levels shown with horizontal bars. The input resistances of the AE cells did not decline after cutting.

cell from region γ on the contralateral AE cell, as shown for the *left* mP cell (mP_c) in Fig. 7*B*. To test the strength of the remaining connection, recordings were made from the sensory and motor cell while stimulating the mP cell before and after cutting, as shown in the left column in Fig. 7*C* and *D*. The mP cell continued to elicit synaptic potentials in the AE cell after cutting, but EPSPs were diminished to $72 \pm 8\%$ ($n = 7$) in the ipsilateral AE cell and $70 \pm 5\%$ ($n = 3$) in the contralateral AE cell. In some records a radiative coupling artifact happened to precede the synaptic potentials (Fig. 7*C*; see Methods). As a control, in twenty-one cases the mP cell anterior axon was cut within the neuropile at the edge of the ganglion near the connectives, without removing secondary branches. The synaptic potential in these AE cells after mP cell axotomy was $111 \pm 6\%$ of that before axotomy, thus if it changed at all, it slightly increased. Overall, the experiments showed that synapses were made in the δ-region of apparent contact between the mP and contralateral AE cells and indicated less directly contact in the γ-region as well.

Although Fig. 7*B* shows the connection between two cut mP cells and a single AE cell, the distribution of contacts can instead be determined between a single P cell and two AE cells, as long as the patterns of connection are bilaterally symmetrical. Thus, the cut of contralateral branches of the mP cell in Fig. 7 also removed the link between the mP cell and its β-contact region on the ipsilateral AE cell, as shown for the *right* mP cell (mP_i) in Fig. 7*B*. Again the synaptic potential persisted, although it was slightly reduced in amplitude, as shown in Fig. 7*D*. This was independent confirmation that region α is a functional synaptic site. The reduction in synaptic potentials recorded in contralateral and ipsilateral AE cells after cutting the P cells' contralateral branches further indicated that synapses form in contact areas β and γ.

Severing mP cell branches and the AE cell axon together

The experiments cutting single cells showed that synapses were made between mP and AE cells in contact regions α, γ and δ (Figs 6 and 7) and provided evidence that contacts in region β were also functional synapses. However, the conclusion was limited by the assumption that the diagrams accurately represent the cells and their contacts. There is a small projection of AE dendrites contralaterally that arises from the ipsilateral axon and could conceivably have made contact with the contralateral mP cell in region δ. This connection would not be expected to be severed by cutting the AE cell axon at the mid-line to remove the contralateral AE arborization, as described above for Fig. 6.

The approach taken to eliminate this possibility was to cut both the mP cell and the contralateral AE cell (Fig. 8). This left only contacts that were made by contralateral branches of the sensory neurone upon the contralateral motor cells; physiological recordings confirmed that synaptic transmission persisted (Fig. 8*C*). Thus region γ is indeed synaptically active. In this series of experiments the synaptic potential dropped to $36 \pm 12\%$ ($n = 8$) of its value before axotomy ($1{\cdot}76 \pm 0{\cdot}03$ mV). When the contralateral branches of the mP cell were cut in addition to the cuts made for Fig. 8, there was no overlap of branches between the two cells, nor was there a detectable EPSP ($n = 7$, records not shown).

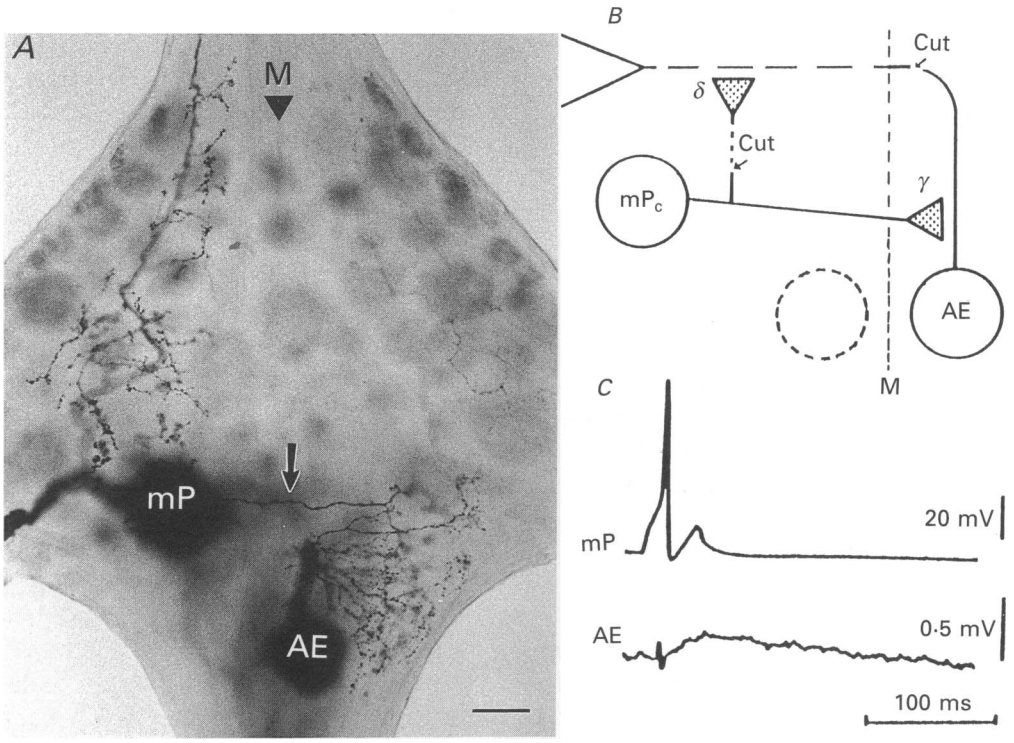

Fig. 8. Transmission from cut mP cells to cut AE cells remains. Each cell was stained with HRP. The entire contralateral portion of each AE cell and most of the posterior axon and associated secondary branches of the contralateral mP cell were axotomized with the laser (*A* and *B*). M indicates ganglion mid-line. Only ipsilateral branches of AE cells and the contralateral branches of mP cells remained, thus only those branches were possible sites of synaptic contact. *B*, only the γ synaptic region remains after the cuts. The neurones were studied electrophysiologically prior to injection with HRP or LY dye. *C*, an action potential generated in each mP produced a direct EPSP in the AE cell.

DISCUSSION

Impulses in the AE motoneurone arise at the primary bifurcation

In the sensory–motor reflex the activity of motoneurones reflects the integration of synaptic inputs at an action potential initiation zone. The interactions of inputs may be affected by their spatial distribution as well as their distance from the impulse initiation site, which in vertebrates is the axon hillock-initial segment (Eccles, 1964; Waxman & Quick, 1978; Kuffler *et al.* 1984). In contrast, invertebrate motoneurones are unipolar and action potentials in many may arise at a site distant from the soma (Stuart, 1970; Pitman *et al.* 1972; Pearson & Fourtner, 1975; Heitler & Goodman, 1978; Granzow *et al.* 1985). Typically the soma and proximal axon are not electrically excitable. The experiments in this study, combining laser microsurgery and electrophysiology, demonstrated that impulses in the AE cell arise at

a single site, at or near the primary bifurcation. Separate lines of evidence from laser axotomy and the timing of impulse propagation indicated that the region between the branch point and the soma of the neurone is inexcitable. The numerous branches arising from this segment of the primary axon of the AE cell are sites of synapses. Since the branches are postsynaptic rather than presynaptic (Muller & McMahan, 1976) they function as dendrites.

Another motoneurone in the leech, the L cell which excites the longitudinal musculature, has been cut using laser axotomy with a similar result; impulses are initiated at the primary bifurcation (X. Gu, unpublished). Such laser axotomy experiments have also been done in a different postsynaptic neurone, the anterior pagoda (AP) cell, whose function is unknown but which has a structure very similar to that of the AE and L motoneurones. The soma and proximal segment of the primary axon of the AP cell are inexcitable while its primary axon, beginning about 100 μm proximal to the bifurcation, is excitable, for cutting distally to that point still permitted impulses to be generated (X. Gu, unpublished). Consistent with having a region of axon closer to the soma that is excitable, electrotonically attenuated impulses recorded in the AP soma were typically twice the size of those recorded in the AE and L somata, in which impulses spread electrotonically from their axons' principal bifurcation. Time differences in the propagation delay of impulses were measured in one AP cell, in which impulses propagated at an average speed of 0·45 m/s. From measured time differences, the impulse initiation zone in that AP cell was calculated to be about 70 μm proximal to the primary axon bifurcation, which nearly matches the location estimated in the laser cutting experiments (X. Gu, unpublished).

Previous experiments in leech ganglia used crushes to detach the contralateral portion of the axon and distal arbor of an inhibitory motoneurone and thereby eliminate the neurone's excitability, presumably removing the impulse initiation zone (Granzow *et al.* 1985). This suggested that the impulse initiation zone was contralateral in the ganglion, in agreement with the results presented here.

The somata of unipolar motoneurones in some invertebrates are excitable (Kandel, 1976; Krauthamer & Ross, 1984), but in the leech and many other species they are not. For example in arthropods there is evidence for a differential distribution of voltage-gated sodium channels, which changes to include the soma after axotomy (Pitman *et al.* 1972; Goodman & Heitler, 1979; Kuwada, 1981; Roederer & Cohen, 1983). A similarly enhanced somatic excitability occurs over days or weeks in leech motoneurones plated in tissue culture medium (Fuchs, Nicholls & Ready, 1981) and in goldfish Mauthner cells axotomized *in vivo* (Titmus & Faber, 1986), where a shift in the distribution of voltage-gated sodium channels has been demonstrated. Evidence from ligand binding studies confirms physiological studies and indicates that sodium channels are typically concentrated at the axon hillock-initial segment of vertebrate neurones (Angelides, Elmer, Loftus & Elson, 1988).

Diverse motoneurone branches can receive input from a single presynaptic cell

The AE cell receives synaptic inputs from various sources, including sensory neurones, interneurones and other motoneurones in the leech ganglion. Synapses on the neurone are electrical and chemical, excitatory and inhibitory. The AE cell sends branches from its main axon both ipsilaterally and contralaterally, forming two

separate regions of dendritic arborization. Both medial P cells synapse with the AE cell in both regions, thus they share the same broad pattern of innervation. Although light microscopic examination of doubly stained preparations reveals that there are multiple sites of possible contact on both sides, the present results do not permit higher resolution of the distribution of functional contacts. Nevertheless, it is clear that for leech motoneurones the inputs from each sensory cell are distributed widely on various dendrites.

There has been a question of whether secondary branches of sensory neurones in the leech ganglion are capable of conducting action potentials, particularly in those long branches that cross the ganglion. Experiments using voltage-sensitive dyes (Krauthamer & Ross, 1984) suggest that secondary branches in barnacles might be excitable. In the present experiments in which sensory and motoneurones were both cut with the laser, contralateral branches of the P cell were identified as sites of functional synapses. Previous experiments using conduction block to study synaptic transmission from leech mechanosensory neurones (Muller & Scott, 1981; Macagno *et al.* 1987) showed that there is no measurable transmission without active invasion of the terminal by an action potential. One must conclude that the secondary branches of leech mechanosensory neurones usually conduct action potentials.

Synaptic integration in the AE cell

The AE and L cells receive significant excitatory input from sensory neurones involved in reflexive activation of the annulus erector and longitudinal musculature in the leech body wall (Nicholls & Purves, 1970; Muller & Nicholls, 1974; Jansen *et al.* 1974). These cells are active when the leech shortens in response to poking. Input from the sensory neurones is gathered by motoneurone secondary branches, which are not themselves presynaptic (Muller & McMahan, 1976). Although there might be local postsynaptic interactions between excitatory and inhibitory inputs, signals are expected to be passively channelled to the main axon and thence to the primary bifurcation. The presumed dearth of voltage-gated sodium channels in the axon proximal to the bifurcation would be expected to increase the axon's space constant and thereby reduce shunting during depolarization by EPSPs. If the space constant of the primary axon were similar to that for the axon of the S cell in the leech, which is greater than 3 mm (Muller & Carbonetto, 1979), synaptic potentials from both sides of the ganglion could interact at the axon bifurcation.

This situation contrasts with that for certain neurones that have electrically excitable dendrites, such as Purkinje cells in the cerebellum, which might appear to offer little opportunity for direct interaction between spatially distant inputs (but cf. Chan, Hounsgaard & Midtgaard, 1989; Hounsgaard & Midtgaard, 1989*a*, *b*). Leech motoneurones may resemble vertebrate motoneurones in integrating passively spreading inputs at a single initiation site. However, the inputs from a single sensory afferent may be restricted to one or a few neighbouring dendrites in the vertebrate (Brown & Fyffe, 1981; Lichtman *et al.* 1984; Purves & Lichtman, 1985) instead of the larger dendritic fields seen in leeches. It has been argued that competition during development segregates synapses to different dendrites in the vertebrate (Purves & Lichtman, 1985). The distribution of synapses formed between the mP cells and the AE cell would suggest that competition might not play that role in the leech.

The L motoneurones in the leech are electrically coupled and fire together to shorten the leech symmetrically, so that it does not bend to the left or right. In contrast, the AE motoneurones are only weakly coupled, so that an impulse in one does not produce an impulse in the other. The arrangement of synapses of the two medial P cells on each AE cell leads to a balance of synaptic inputs from these two cells, so that each AE cell in a ganglion receives similar strength inputs when either medial P cell is firing. This is consistent with the observed behaviour of the animal. Each annulus erects as a ring when the animal is poked on either side, reflecting the concerted reflexive activity of both AE cells.

We thank A. Morrissey and R. Harris for technical assistance, D. McCulloh for writing the data acquisition and analysis programs, and E. McGlade-McCulloh for useful discussions. Work performed by X. Gu was in partial fulfilment of requirements for a Ph.D. Supported by USPHS Grant RO1-NS20607.

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