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SWIM INITIATION IN THE LEECH BY SEROTONIN-CONTAINING INTERNEURONES, CELLS 21 AND 61

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SUMMARY

1. Two pairs of serotonin-containing neurones, designated cells 21 and 61, were characterized physiologically and anatomically in the hirudinid leeches *Macrobdella decora* and *Hirudo medicinalis*. Both of these cells are bilaterally paired interneurones and each cell is weakly electrically coupled to the other serotonin-containing cells both intra- and interganglionically.

2. Cells 21 and 61 are excited polysynaptically by individual identified mechanosensory neurones. Segmental nerve shock sufficient to elicit an episode of swimming strongly excites cells 21 and 61, which then tend to generate bursts of impulses that are phase-locked to the swim motor pattern.

3. Intracellular stimulation of either cell 21 or cell 61 often causes the initiation of swimming, acting in parallel with the nonserotonergic swim-initiator cell 204. Cells 61 and 204 are also weakly electrically coupled.

4. The latency to swim onset by stimulating cell 21 or 61 is similar to that of cell 204 and different from that of the serotonergic Retzius cell. This result, with those in the accompanying paper (Nusbaum, 1986), suggests that unlike the Retzius cell and similar to cell 204, cells 21 and 61 synaptically contact cells of the swim central pattern generator (CPG).

INTRODUCTION

Biogenic amines influence the motor output underlying many types of behaviour in both vertebrates and invertebrates. For instance, among the vertebrates, exogenously applied amines often initiate or enhance the excitation of motor activity (Grillner, 1975; Shik & Orlovsky, 1976; Poon, 1980; White & Neumann, 1980; Vander Maelen & Aghajanian, 1982). Exogenously applied amines also initiate rhythmic motor activity in many invertebrates (Granzow & Kater, 1977; Anderson & Barker, 1981; Willard, 1981; Mackey & Carew, 1983) and can also cause stereotyped behavioural responses (Livingston, Harris-Warrick & Kravitz, 1980; Horvitz *et al.* 1982). Moreover, in the invertebrates, individual identified amine-containing neurones often initiate or modulate rhythmic motor patterns (Mayeri *et al.* 1974;

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Key words: identified serotonin neurones, central pattern generator initiation, leech swimming.

Liebeswar, Goldman, Koester & Mayeri, 1975; Granzow & Kater, 1977; Evans & O'Shea, 1978; O'Shea & Evans, 1979; Gelperin, 1981: Granzow & Rowell, 1981; Kupfermann & Weiss, 1981; Willard, 1981; Claiborne & Selverston, 1984; Marder & Eisen, 1984).

In this paper we present evidence that two identified serotonin-containing neurones, cells 21 and 61, can initiate swimming in the leech. Previously it has been shown that in the leech *Hirudo medicinalis* application of serotonin acts at the periphery, both to effect mucus release (Lent, 1973) and to modulate the contractility and resting tension level of longitudinal muscle (Mason & Kristan, 1982). Centrally serotonin acts to initiate swimming motor activity (Willard, 1981). The isolated leech nerve cord responds with repeated swimming episodes during bath application of $>500\,\text{nmol}\,l^{-1}$ serotonin (5-HT).

The initiation of repeated episodes of leech swimming by exogenously applied serotonin can be mimicked by intracellular stimulation of the serotonergic Retzius cells (Willard, 1981; Arbas & Calabrese, 1984). The Retzius cells apparently influence swimming by increasing the levels of serotonin in the blood, rather than by synapsing directly on the neurones of the swim circuit (Willard, 1981; B. Granzow, W. B. Kristan, M. P. Nusbaum & J. C. Weeks, unpublished observations).

The Retzius cells are found in all segmental ganglia (Lent, 1982; Fig. 1). There are 4–7 additional serotonin-containing neurones per ganglion (Lent, 1982; Glover, 1984). Four of these neurones, occurring as two bilaterally symmetrical pairs of lateral somata designated cells 21 and 61, are found in nearly every segmental ganglion (Lent, 1982; Kristan & Nusbaum, 1983; Glover, 1984; Fig. 1). Other serotonin-containing neurones are found only in some of the ganglia and are apparently not necessary for swimming (Glover, 1984; Nusbaum, 1984).

This paper presents an anatomical and physiological characterization of cells 21 and 61, and their synaptic interactions with both the Retzius cell and cell 204. These data provide strong evidence that cells 21 and 61 function in swim-initiation in the leech. Some of these results have previously been published (Nusbaum & Kristan, 1982; Nusbaum, 1983; Kristan & Nusbaum, 1983).

MATERIALS AND METHODS

Leeches, *Macrobdella decora*, obtained from St Croix Biological (Stillwater, MN), and *Hirudo medicinalis*, obtained from Blutegelimport und Versand (Elisabethstrasse 16a, West Germany), were maintained at 15°C in aquaria as described previously (Weeks, 1981). The majority of the experiments were performed on *Macrobdella decora*. Except where noted, similar results were also obtained with *Hirudo medicinalis*. Leeches were fed at least once every 8 weeks on fresh, heparinized bovine blood through the walls of bovine intestines.

Dissections and experiments were performed in leech physiological saline, containing: (in $\text{mmol}\,l^{-1}$) NaCl, 115; KCl, 4; $CaCl_2$, 1·8; $MgCl_2$, 1·5; glucose, 10; Tris maleate, 4·6; Tris base, 5·3; pH 7·4. Many experiments were often performed with a slightly elevated Mg^{2+} concentration of $3–5\,\text{mmol}\,l^{-1}$ (Weeks, 1981). To

determine whether synaptic interactions were chemical or electrical, neurotransmitter release was reversibly depressed using saline with either an elevated Mg^{2+} concentration (10 mmol l^{-1}) and a reduced Ca^{2+} concentration (0·45 mmol l^{-1}), or a higher concentration of Mg^{2+} (25 mmol l^{-1}) and the normal concentration of Ca^{2+} (1·8 mmol l^{-1}) (Nicholls & Purves, 1970). To test its effectiveness in depressing leech monosynaptic chemical synapses, the 10 mmol l^{-1} Mg^{2+}/0·45 mmol l^{-1} Ca^{2+} saline initially was shown to inhibit reversibly both the direct chemical inhibition of cell 3 by cell 1 (Granzow, Friesen & Kristan, 1985) and the direct excitation of cell 208 by cell 204 (Weeks, 1982*b*). However, some chemical synapses in the leech are only partially suppressed with these elevated levels of Mg^{2+} (Nicholls & Wallace, 1978), and therefore the possibility remains that synaptic interactions surviving these conditions may still have a chemical component. Most experiments were performed with darkfield illumination of desheathed ganglia (Friesen, 1985). All experiments were done at 20–23 °C.

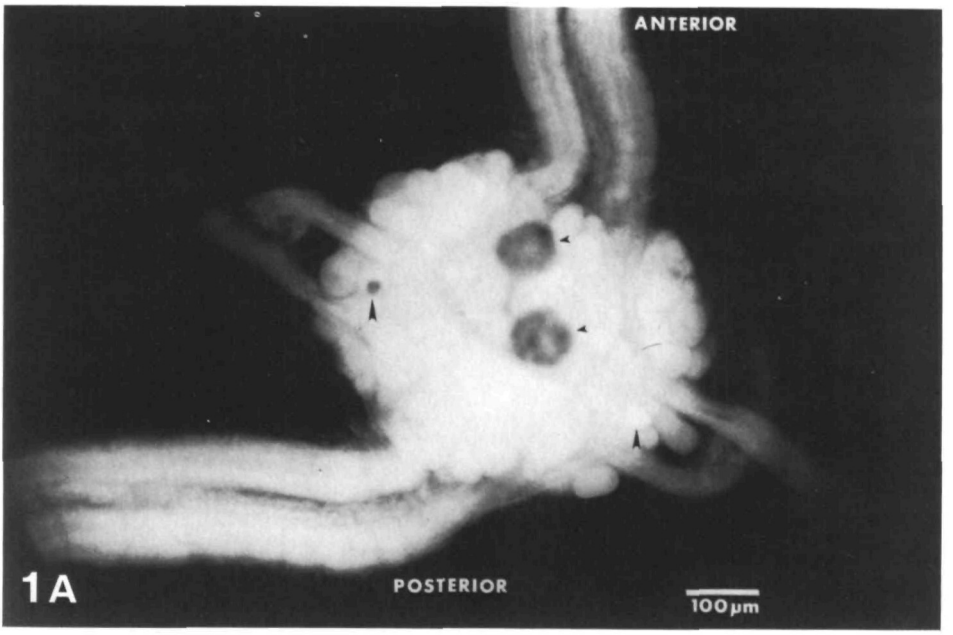
The electrophysiological techniques used both for intracellular and extracellular recording and for stimulating neuronal activity were as previously described (Kristan, Stent & Ort, 1974; Ort, Kristan & Stent, 1974). Amplified recordings were stored on magnetic tape for later playback using a Vetter Crown model A tape recorder. In many experiments, the fluorescent dye Lucifer Yellow (Stewart, 1978) was injected intracellularly by iontophoresis to determine the pattern of arborization and to verify the identity of neurones studied physiologically.

Focal application of serotonin was made using a serotonin-filled micropipette attached to a pressure ejection device. Serotonin (10^{-7}–10^{-3} mol l^{-1}) was dissolved in physiological saline (pH 7·4) and ejected from broken-tipped micropipettes (30–70 MΩ) using positive pressure (14–200 kPa).

Identification of cells 21 and 61

The dye Neutral Red stains selectively the somata of serotonin-containing neurones in the leech CNS in both living and fixed preparations (Stuart, Hudspeth & Hall, 1974; Lent, Ono, Keyser & Karten, 1979; Glover, 1984). The somata of the serotonin-containing neurones can be readily located following a brief application (2–10 min) of 0·0005 % Neutral Red to isolated nerve cords, or by soaking an intact leech in 0·0005 % Neutral Red for at least 12 h prior to dissection (Lent, 1981) (Fig. 1A). The latter technique consistently gave less background staining. Neutral Red can also photoinactivate neurones in leech ganglia, including those neurones that are not obviously stained red by the dye application (A. Mason, W. B. Kristan, W. O. Friesen & M. P. Nusbaum, unpublished observations), so electrophysiological recordings were routinely performed before exposing ganglia to Neutral Red, or with minimal exposure to light.

Cells 21 and 61 were identified during electrophysiological recordings on the basis of their soma position and size, plus a number of physiological properties as described in the Results section of this paper. In initial experiments, following recordings the putative serotonin-containing cells were filled with Lucifer Yellow, after which the ganglion was exposed to Neutral Red to confirm that the Lucifer



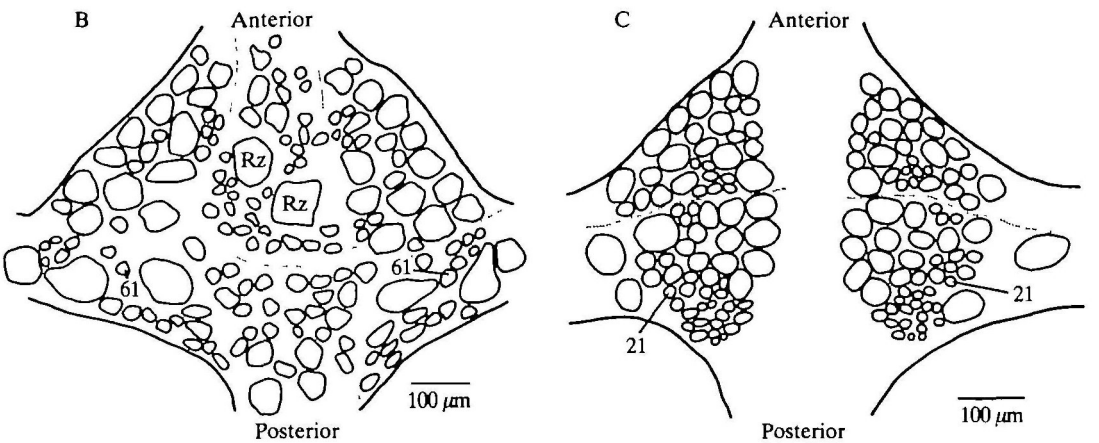


Fig. 1. (A) Photomicrograph of the ventral surface of a segmental *Macrobdella* ganglion stained with 0·0005 % Neutral Red. The Neutral Red-stained somata of the pair of Retzius cells (arrowheads; large cells) and one of the two cells 61 (left, arrowhead; small cell) appears darker than the surrounding unstained somata. The cell 61 soma on the right side of the ganglion (right arrowhead, small cell) was filled with Lucifer Yellow and therefore appears lighter than the surrounding somata. The ganglion, viewed in unfixed wholemount, was illuminated with transmitted white light and epifluorescence, to highlight both the Neutral Red staining and the Lucifer Yellow fluorescence. When this ganglion was illuminated with only white light, the Neutral Red stain appeared in the Lucifer Yellow-filled cell 61 as well. Unless otherwise noted, all figures are from *Macrobdella*. (B),(C) Schematic maps of the ventral and dorsal surfaces of a *Hirudo* segmental ganglion. Cells 61 and the Retzius cells are indicated on the ventral surface (B) and cells 21 are indicated on the dorsal surface (C). Labelling scheme according to Ort, Kristan & Stent (1974) and Muller, Nicholls & Stent (1981). These *Hirudo* maps can be used for *Macrobdella* with minor modifications not relevant to cell 21, cell 61 or the Retzius cell.

Yellow-filled cell was a serotonin-containing cell (Fig. 1A). After dozens of such experiments gave consistent results, identification of cells 21 and 61 was made only by their unique structures when filled with Lucifer Yellow, or by confirming that the recorded cell later stained with Neutral Red.

Serotonin, Neutral Red and Lucifer Yellow were obtained from the Sigma Chemical Company; some of the Lucifer Yellow was a gift from Walter Stewart.

Terminology

In this paper, the terms 'swimming' and 'swim' will be used as a shorthand notation to indicate 'the motor programme for swimming' or 'bursting in nerves characteristic of swimming'.

RESULTS

There is a single pair of bilaterally symmetrical serotonin-containing neuronal somata on the ventrolateral surface of each leech segmental ganglion (Lent, 1982; Glover, 1984). This pair of cells has been designated cell 61 in accordance with its stereotyped location within the ganglion and the previously constructed scheme for labelling individual somata in these ganglia (Ort *et al.* 1974; Muller, Nicholls & Stent, 1981) (Fig. 1B). Similarly, the single pair of serotonin-containing neurones whose somata are located dorsolaterally in the ganglia has been designated cell 21 (Fig. 1C).

Intracellular injections of Lucifer Yellow showed that the intraganglionic arborizations of cells 21 and 61 were each unique, as is true for many leech neurones (Muller *et al.* 1981) (Fig. 2). Although similar, cells 21 and 61 were readily distinguishable from one another. Both had a single, relatively large neurite extending from the soma directly across to the contralateral side of the ganglion. Furthermore, both cells had an extensive cluster of small processes extending out of the main neurite, primarily within the ipsilateral neuropile. These arbors extended both dorsally and ventrally throughout the neuropile, in contrast to the arbor of some other identified swim-related interneurones which are restricted primarily to the dorsal neuropile (Weeks & Kristan, 1978; Weeks, 1982*b*). On the contralateral side of the ganglion, the primary neurite of cell 61 always bifurcated in a characteristic 'T' shape ($N > 50$), whereas the corresponding bifurcation of cell 21 was always 'Y' shaped ($N > 20$). The two major contralateral branches of cell 61 had numerous smaller processes that ramified within the contralateral neuropile. In contrast, there were very few processes from the contralateral axons of cell 21. Both cell types sent axons into both anterior and posterior contralateral connectives; the axon of cell 61 was always medial to that of cell 21. No processes of either cell were ever found to enter any peripheral segmental nerve.

Intracellular recordings from the somata of cells 21 and 61 typically gave resting potentials of -40 to -50 mV. Recorded impulses were only 3–10 mV, presumably because they were propagated passively into the somata (Muller, 1981). These cells showed small afterpotentials following impulses, with a duration often as much as

60–80 ms (see Fig. 3). In the absence of any stimulation of the nerve cord, cells 21 and 61 were either inactive or fired spontaneously at frequencies of 0·5–4 Hz. For both cells 21 and 61, each intracellular impulse was followed at a fixed latency by extracellularly recorded spikes in the interganglionic connectives no further than two segments in either direction (Fig. 3), suggesting that these cells extend processes only to the two nearest ganglia in each direction. No time-locked spikes were recorded from the peripheral nerves of these neighbouring ganglia so apparently neither cell type extends processes to the periphery in any segment.

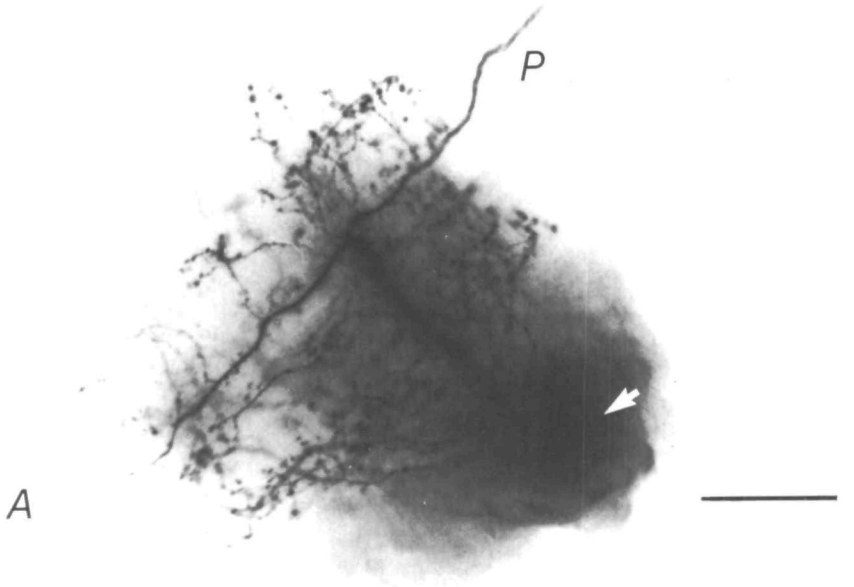
Electrical coupling

An earlier, qualitative analysis of the interactions among Neutral Red-stained serotonin-containing neurones in the leech *Haemopis marmorata* reported sufficiently strong electrical coupling among these cells to produce simultaneous impulse bursts (Lent & Frazier, 1977). Therefore, a quantitative analysis of these interactions, in unstained preparations, was undertaken to determine the efficacy of this coupling in *Macrobdella*. Individual cells 21 and 61 were impaled with two microelectrodes to independently inject current and record their membrane potential, while simultaneously recording intracellularly from another homoganglionic serotonin-containing cell.

Injecting either depolarizing or hyperpolarizing current pulses into an individual cell 21 or 61 caused a membrane potential change of similar sign but reduced amplitude in its contralateral homologue, as well as in each of the cells of the other lateral serotonin-containing cell pair and in the Retzius cells (Fig. 4). This apparently nonrectifying electrical interaction was weak and was retained in saline containing elevated Mg^{2+} (25 mmol l^{-1}) or elevated Mg^{2+} (10 mmol l^{-1}) and reduced Ca^{2+} (0·45 mmol l^{-1}) (e.g. Fig. 5). The coupling coefficient for hyperpolarization between bilateral homologues of cells 21 and 61 was approximately 0·03 (range 0·02–0·06), when determined from the slope of a plot of the change in presynaptic membrane potential *vs* postsynaptic membrane potential. Similar coupling coefficients were found for the interactions between cells 21 and 61 and the other lateral serotonin-containing cell pair, and the Retzius cells.

As a test of the functional strength of the interactions among these cells, the relative change in impulse frequency was measured from pairs of these serotonin-containing neurones. Injecting depolarizing current (0·2–2·0 nA) into a cell 21 or 61 so that it fired at frequencies (0·5–20 Hz) seen during normal behaviour weakly depolarized and increased the impulse activity in the other serotonin-containing cells (Fig. 4). During these depolarizing current injections, unlike the case reported by Lent & Frazier (1977), simultaneous impulse bursts were never observed in the coupled serotonin-containing cells. When a cell 21 or 61 was stimulated to levels of

Fig. 2. Photomicrographs of Lucifer Yellow-filled cells 61 (A) and 21 (B). (A) The anterior (*A*) and posterior (*P*) interganglionic connectives are to the lower left and upper right, respectively. (B) The anterior and posterior interganglionic connectives are to the left and right, respectively. Arrows indicate the position of the dye-filled somata. Both photographs are negative images of unfixed wholemounts. Scale bars, 100 μm.



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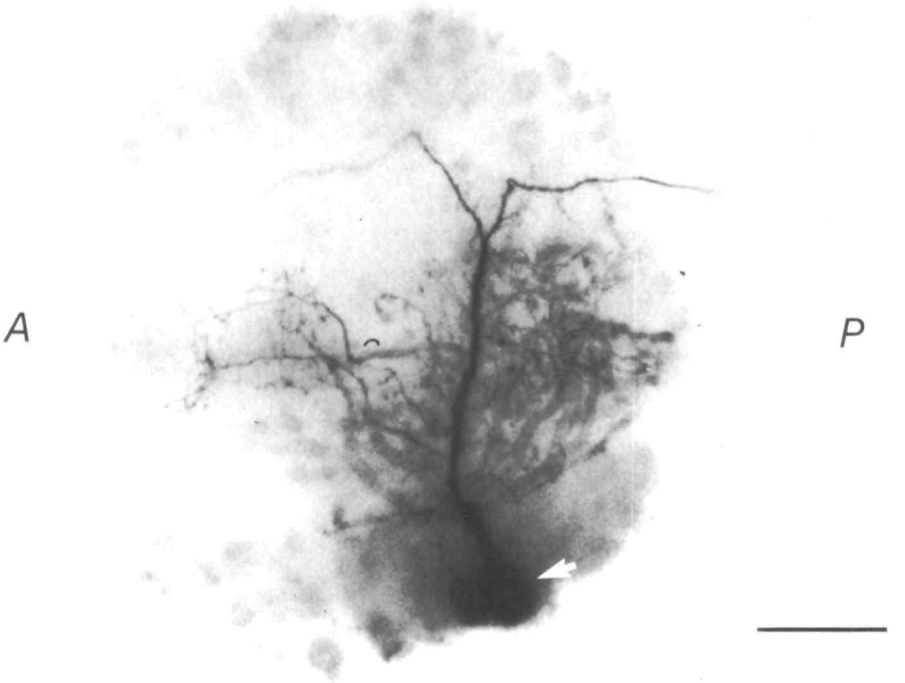

B

impulse activity capable of initiating swimming (10–20 Hz), the change in the impulse frequency of the postsynaptic cell was only approximately one-tenth that produced by the presynaptic cell. Such stimulation of cells 21 or 61 caused a slight depolarization (<2·0 mV) and a maximum impulse frequency of 1 Hz in the coupled cell if this cell was initially below impulse threshold. If the coupled cell was already active, its activity was increased by 0·5–3 Hz. The strength of the effect on the firing frequency of the postsynaptic cell was greater when cell 21 or 61, instead of the Retzius cell, was the postsynaptic cell. Depolarizing either cell 21 or cell 61 also excited all of the serotonin-containing cells in the neighbouring two ganglia both anteriorly and posteriorly (e.g. Fig. 5E). These interactions among cell 21, cell 61 and Retzius cells were similar in *Hirudo* and *Macrobdella*.

Fig. 5A shows an interesting feature of the interaction of cell 61 and the Retzius cell. Each impulse in cell 61 caused an excitatory postsynaptic potential (EPSP) of 0·5–1 mV in the Retzius cells within the same ganglion, with a delay of 5 ms. This EPSP persisted with unchanged amplitude in an altered saline (25 mmol l^{-1} Mg^{2+}) that blocked many other known monosynaptic chemical interactions (Nicholls & Purves, 1970; M. P. Nusbaum, unpublished observations) and partially suppressed all others (Nicholls & Wallace, 1978). Furthermore, the EPSP amplitude did not change when the membrane potential of the Retzius cell was either depolarized or hyperpolarized. These results suggest that this EPSP could be the electrical coupling

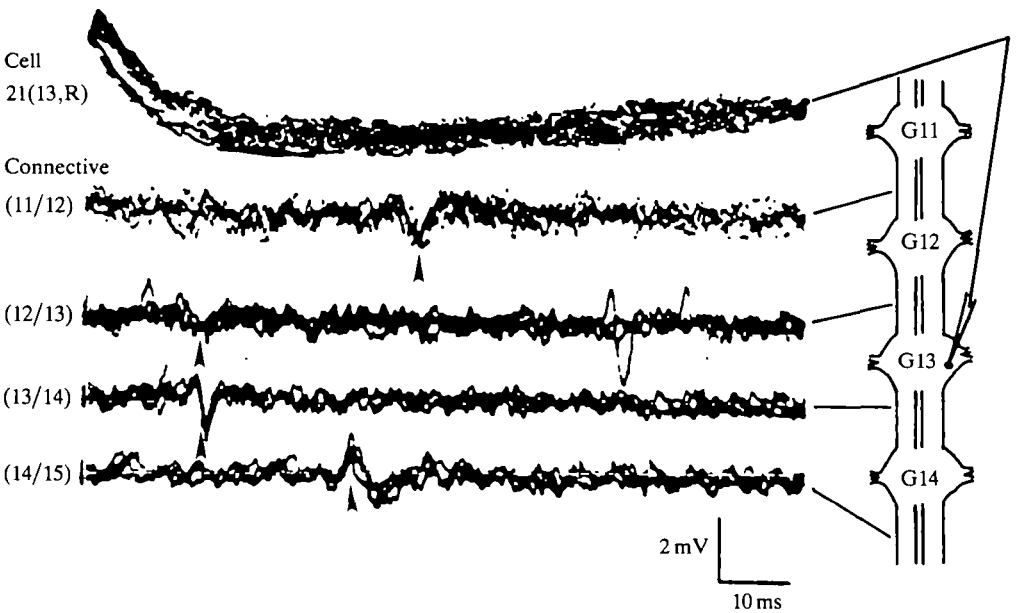

Fig. 3. Interganglionic extent of cell 21. Three superimposed oscilloscope sweeps are shown, each triggered by an impulse in 21(13,R) (top trace). Impulses time-locked to the intracellularly recorded cell 21 impulse were recorded extracellularly from the connective (arrowheads) between ganglia 11/12, 12/13, 13/14 and 14/15 (bottom four traces, respectively). To the right: schematic representation of ganglia 11–14, indicating the recording sites.

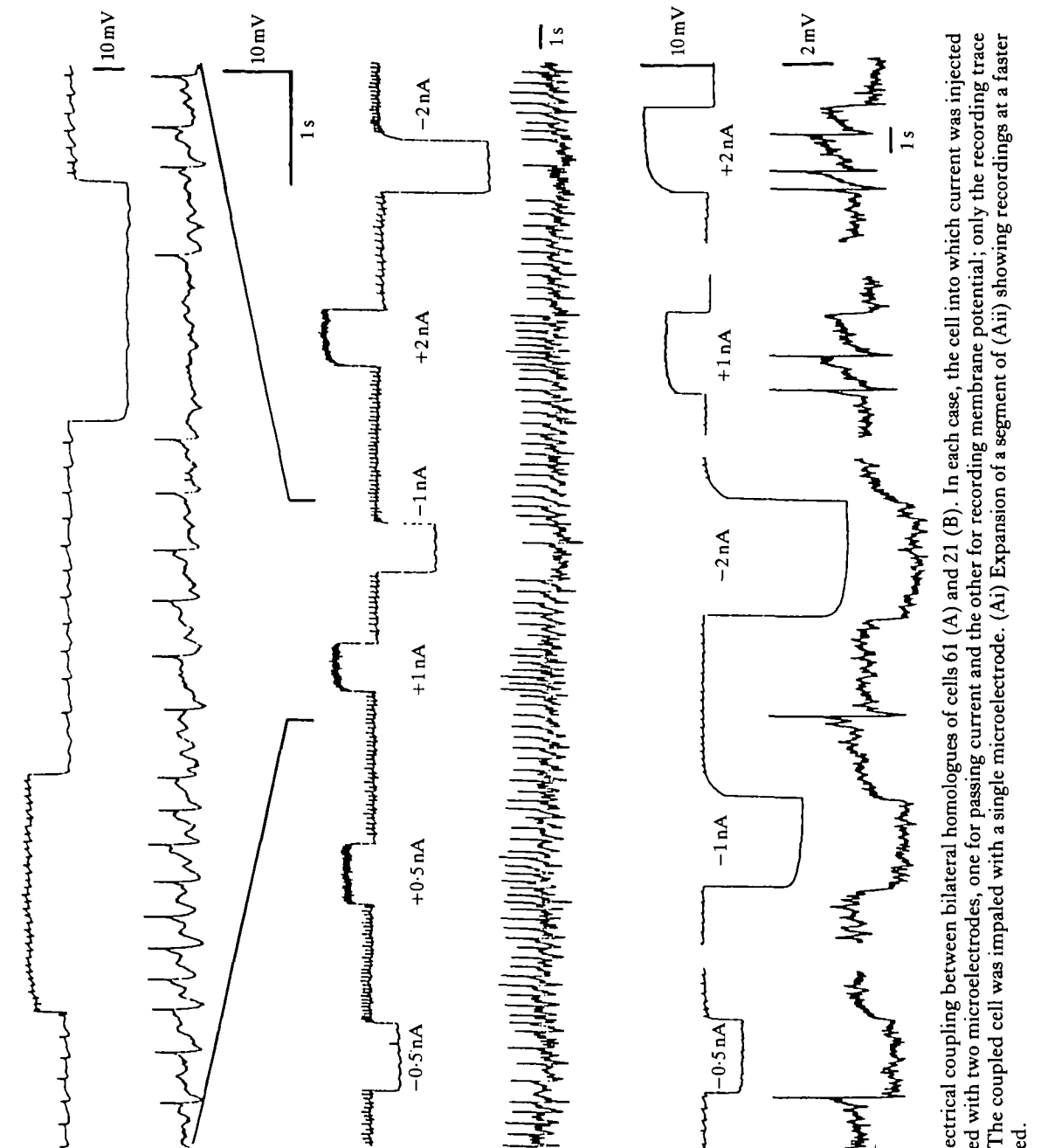

Fig. 4. Electrical coupling between bilateral homologues of cells 61 (A) and 21 (B). In each case, the cell into which current was injected was impaled with two microelectrodes, one for passing current and the other for recording membrane potential; only the recording trace is shown. The coupled cell was impaled with a single microelectrode. (Ai) Expansion of a segment of (Aii) showing recordings at a faster sweep speed.

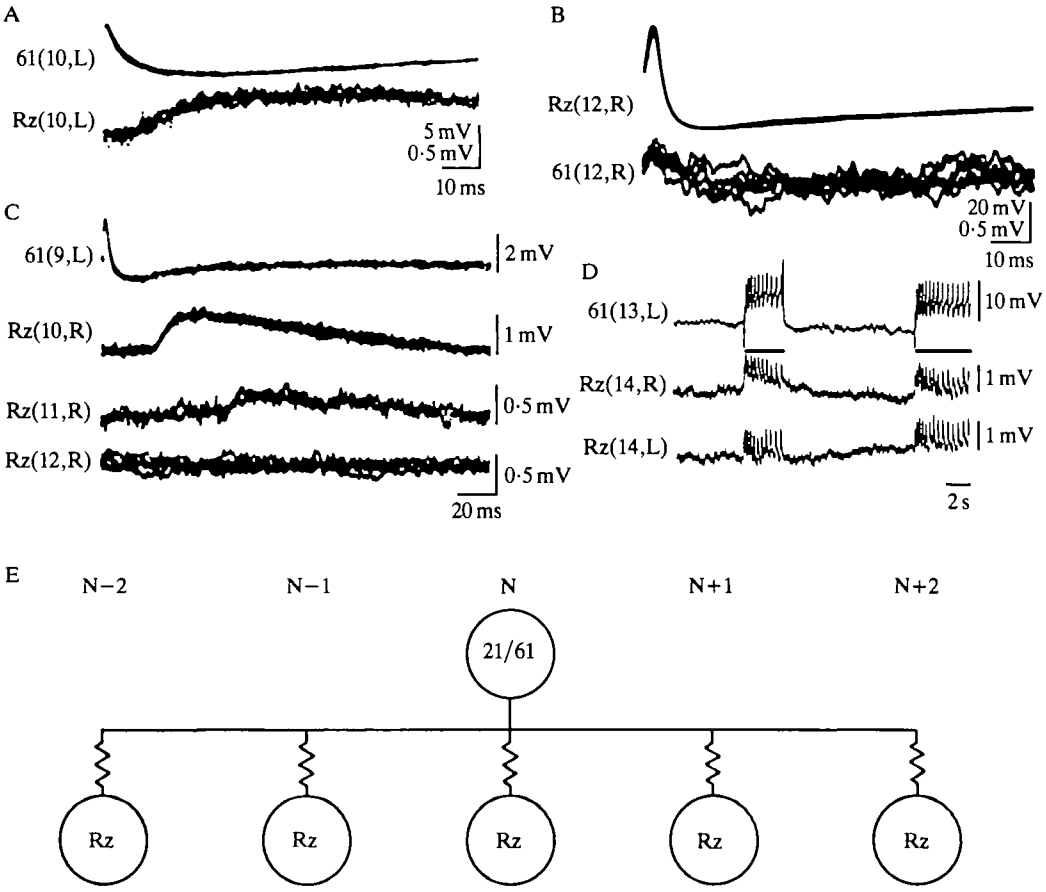

Fig. 5. Intra- and interganglionic synaptic interactions of cell 61 and the Retzius cell (Rz). All recordings are intracellular, and were made in 25 mmol l^{-1} Mg^{2+} saline. (A)–(C) Each oscilloscope sweep was triggered by an impulse recorded in the top cell of each set of recordings. (A) Four superimposed oscilloscope sweeps. (B) Five superimposed oscilloscope sweeps. (C) Composite set of recordings of cell 61(9,L) with a Retzius cell in each of the three neighbouring posterior ganglia; from a succession of three paired intracellular recordings. (D) Impulses in cell 61(13,L) evoked by intracellular depolarization (bars) elicited time-locked EPSPs in both Retzius cells in the next posterior ganglion. (E) Schematic model of the synaptic interactions of cell 61 (N) with the Retzius cell in both the same (N) and nearby ganglia (N+1; N−1; N+2; N−2). All of the indicated synaptic interactions occur bilaterally. Resistor symbols indicate nonrectifying electrical synaptic interactions.

potential from cell 61 to the Retzius cell. The EPSP elicited in cell 61 by Retzius cell impulses was less than 0·5 mV and had no measurable synaptic delay (Fig. 5B). The excitatory response of the Retzius cells to activity in cell 61 was also observed in the two neighbouring anterior (not shown) and posterior ganglia, but not in more distant ganglia (Fig. 5E). Individual EPSPs were not observed in intracellular recordings of cell 21 in response to impulses in the Retzius cell.

The coupling between serotonin-containing neurones was too weak, except between the two Retzius cells, to cause any apparent impulse synchrony, or to

produce bursts of impulses in the postsynaptic cells. Therefore, its major functional significance appears to be a contribution to the tendency for these neurones to be active or inactive at the same time.

Responses of single neurones to serotonin

Exogenous serotonin was applied to cells 21 and 61 and the Retzius cell to determine whether they were one of the loci of the swim-initiating effects of serotonin. No excitatory effects of exogenously applied serotonin were observed, however, on these serotonin-containing cells in the absence of swim-initiation. Instead, cells 21 and 61 (Fig. 6) and the Retzius cell (Kerkut & Walker, 1967; Henderson, 1982; Fig. 6) hyperpolarized in response to pressure ejected application of 10^{-4} mol l^{-1} serotonin either to their somata or into the neuropile of the ganglion containing their somata. This hyperpolarization occurred in response to a serotonin

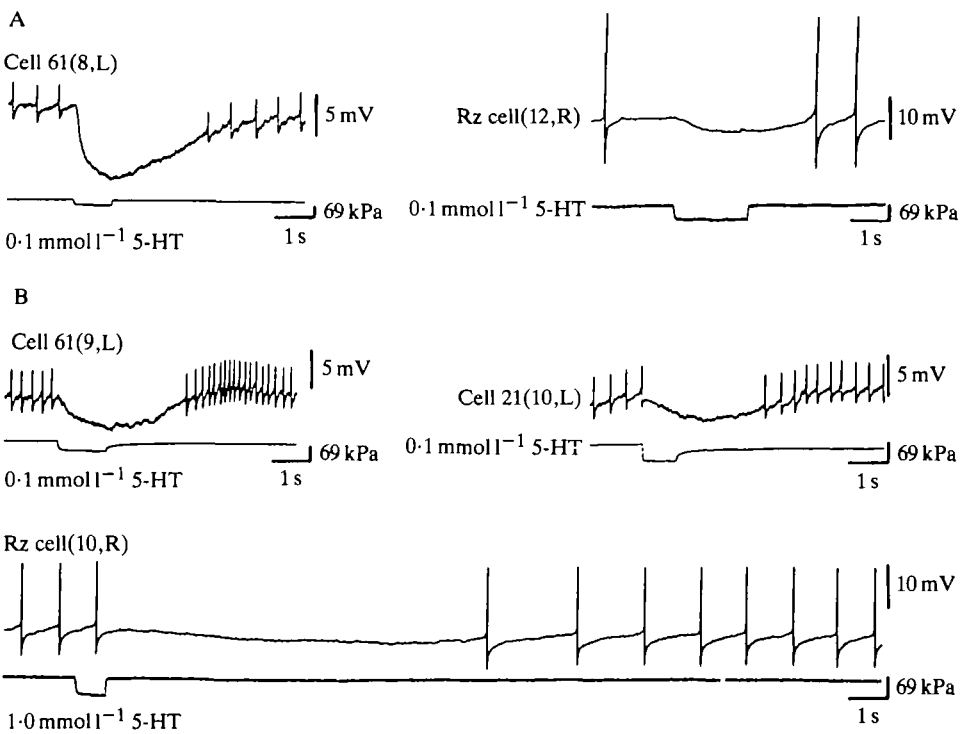

Fig. 6. Responses of serotonin-containing cells to focal application of serotonin. All recordings are intracellular. (A) Responses of cell 61 and the Retzius cell (Rz) to pressure ejection of 0·1 mmol l^{-1} serotonin (5-HT) onto their desheathed somata. (B) Responses of serotonin-containing neurones to pressure ejection of 0·1 mmol l^{-1} serotonin into the neuropile of the desheathed ganglion in which each of the intracellular recordings was made. In each recording, the downward inflection of the 5-HT trace indicates the period of time when serotonin was being pressure ejected. The downward inflection was the result of a voltage change, monitored by an intracellular recording amplifier, occurring across the tip of the pressure pipette due to a current created by pressure ejecting the serotonin solution.

concentration as low as 10^{-7} mol l^{-1}, the lowest concentration tested. Control applications of saline elicited no response from these cells. Individual isolated Retzius cells in culture also hyperpolarize in response to exogenously applied serotonin (Henderson, 1982; M. P. Nusbaum, unpublished observations). Bath application of serotonin gave the same results as pressure ejection. The soma and neuropile responses were apparently due to the interaction of serotonin with receptors on these cells, and not by way of other cells, since (1) these responses still occurred when neurotransmitter release was depressed using saline with elevated Mg^{2+} (10 mmol l^{-1}) and reduced Ca^{2+} (0·45 mmol l^{-1}); (2) the response increased in latency and decreased in amplitude as the pressure pipette was backed away from the soma, either in the direction away from the ganglion or along the surface of the ganglion; (3) no response occurred when the pressure pipette was more than two soma diameters removed from the recorded cell and (4) the responses to pressure ejection of serotonin into the neuropile were localized; moving the pressure pipette a short distance (less than 20 μm) from a site causing a response would often eliminate the response.

When cell 61 was hyperpolarized by application of serotonin to its soma, both Retzius cells in the same ganglion also exhibited hyperpolarization, presumably *via* the electrical junction between these cells. Hyperpolarizing responses to serotonin application were also recorded in some other swim-related neurones, while a mixed excitation and inhibition was recorded in one swim CPG neurone; no response was recorded in other swim-related neurones or in many neurones not involved in swimming (Nusbaum, 1984, 1986).

Response to mechanosensory stimulation and nerve shock

Mechanosensory stimulation of the leech body wall in semi-intact preparations that was sufficient to initiate swimming excited cell 21, cell 61 and the Retzius cell. Such mechanosensory stimulation directly activates identified T (touch-sensitive), P (pressure-sensitive), and N (nociceptive-sensitive) mechanosensory neurones (Nicholls & Baylor, 1968), as well as sensillar movement receptors (Brodfuehrer & Friesen, 1984). Intracellular stimulation of T, P and N cells also excited cells 21 and 61 (Fig. 7). P cells caused the strongest excitatory response, and also produced a considerably weaker inhibitory input; T cells caused the weakest excitatory response. All of these effects were found to be polysynaptic.

Some of the sensory neurone-evoked excitation in cell 61 came by way of a monosynaptic excitatory input from a multimodal, unpaired interneurone known as the S cell (Gardner-Medwin, Jansen & Taxt, 1973; Frank, Jansen & Rinvik, 1975), which is excited by sensory neurones (Muller & Scott, 1981; M. P. Nusbaum, unpublished observations). As shown in Fig. 7D, S cell impulses elicited constant- and short- (0 ms) latency EPSPs in cell 61. This effect persisted when neurotransmitter release was depressed, and exhibited rectification (not shown), suggesting this to be an electrical synaptic interaction. Cell 21 and the Retzius cell also received apparently monosynaptic EPSPs from the S cell, but whether these connections were also electrical was not tested. Each S cell is strongly electrically coupled to S cells in

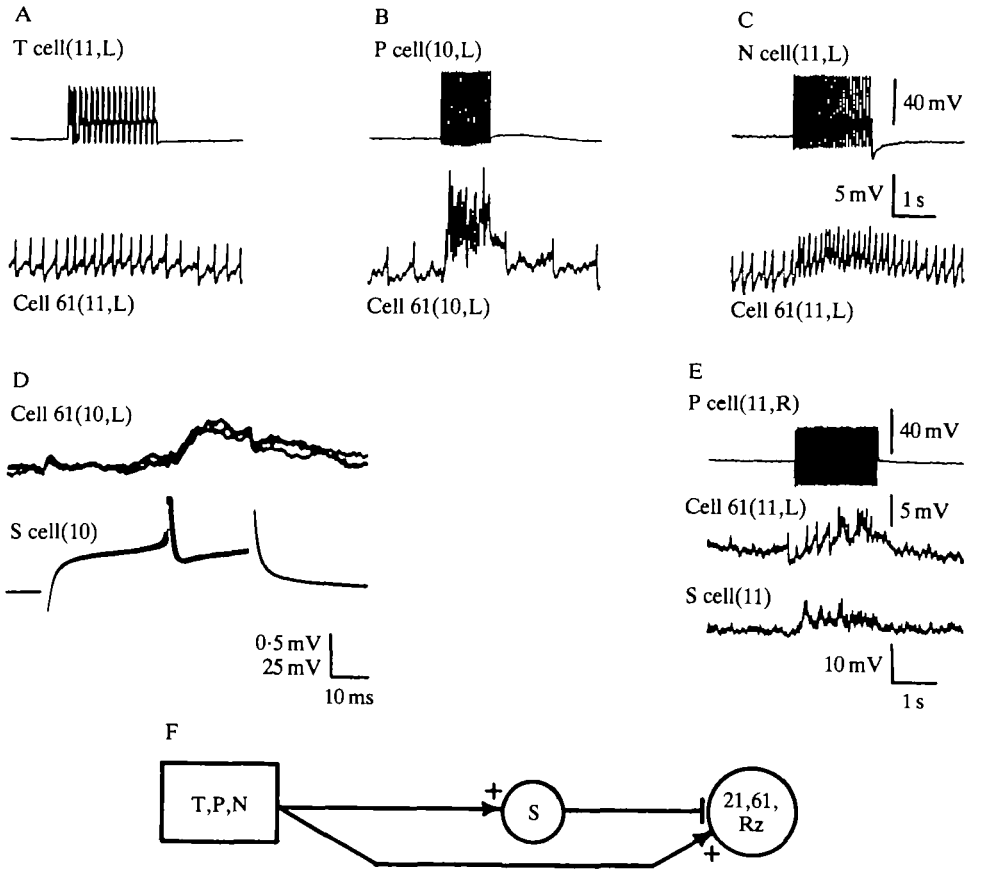

Fig. 7. Pathways of mechanosensory activation of cell 61. All recordings are intracellular. (A)–(C) Simultaneous recordings of cell 61 with a T, P and N cell, respectively, from the same ganglion. In order to determine the firing frequency of the stimulated cell, each sensory cell was impaled with a second intracellular electrode through which depolarizing current was injected (not shown). T, P and N cell firing frequencies were 25 Hz, 16 Hz and 18 Hz, respectively. (D) Three superimposed oscilloscope sweeps of a paired recording of cell 61(10,L) and the S cell(10). The latency of the impulse in the S cell to the EPSP in cell 61 was 0 ms. (E) Simultaneous recording of a P cell, S cell and cell 61 from the same ganglion. Intracellular stimulation of the P cell (approximately 20 Hz) caused a depolarization and a burst of impulses in cell 61 and only a barrage of subthreshold EPSPs in the S cell. (F) Schematic diagram of the pathways for T, P, and N cell mediated excitation of cell 21, cell 61 and the Retzius cell (Rz). 'T' junctions indicate monosynaptic excitation and arrowheads with associated '+' symbols indicate polysynaptic excitation. All synaptic interactions shown here occur bilaterally.

adjacent ganglia, so that S cell impulses in any ganglion are rapidly conducted bidirectionally along the nerve cord. Therefore, activation of the S cell in any segment results in the excitation of cells 21, 61 and the Retzius in all ganglia. However, intracellular stimulation of sensory cells at firing frequencies subthreshold for firing the S cell nevertheless excited cell 61 (Fig. 7E), indicating that the S cell is not the only pathway from the mechanosensory cells to the serotonin-containing cells (Fig. 7F).

Activity during swimming

For cells 21 and 61 to play a role in swim-initiation, they must be active during the interval between the onset of a swim-initiating stimulus and the ensuing swim onset, as are cells 204 and 205 (Weeks & Kristan, 1978; Weeks, 1982*a*) and the Retzius cell (Willard, 1981). During this interval, in response to nerve stimulation resulting in the swim motor pattern, cells 21 and 61 were excited and fired impulses at frequencies up to 20–25 Hz (Fig. 8). In all experiments reported in this paper involving swimming, the swim motor pattern was monitored using extracellular recordings from segmental nerves, in which repeating impulse bursts of identified swim motor neurones can be recorded (Ort *et al.* 1974; Kristan & Calabrese, 1976). During the ensuing bout of swimming, the membrane potential of these serotonin-containing cells weakly oscillated (Fig. 8). Cells 21 and 61 and the Retzius cell depolarized and fired bursts of impulses periodically during swimming which tended to occur in phase with the impulse activity of the homoganglionic excitor motor neurones of the dorsal longitudinal muscles.

Swim-initiation

Stimulation of a single cell 21 or 61 often produced swimming in both semi-intact preparations and in completely isolated, brainless nerve cords (Fig. 9A). Intracellular stimulation of cell 61 caused swim-initiation in 15 out of 30 *Macrobdella* preparations in which swimming was elicited by stimulation of cell 204, a previously described nonserotonergic swim-initiator (Weeks & Kristan, 1978). The impulse frequency (5–20 Hz) attained by such stimulated cells 61 was in the range of frequencies commonly observed in swimming or otherwise active preparations. In nine of the remaining preparations, intracellular stimulation of cell 61 was capable of initiating an abbreviated episode of the swim motor pattern, lasting for no more than three cycles of activity. In the other six preparations, stimulating cell 61 caused only a tonic increase in the activity level of the swim motor neurones. The ability of cell 61 to initiate swimming in *Hirudo* appeared to be less reliable (4 out of 11 preparations). The swim-initiating ability of cell 21 was less exhaustively studied but appeared to be similar to that of cell 61. Swimming sometimes ended before the termination of stimulation of cell 21 or 61, possibly because accommodation of these cells often occurred during long-duration (>10 s) or after repeated current injections. Swim initiation could also be demonstrated when cell 61 (or cell 21) and cell 204 were simultaneously stimulated to fire at levels otherwise inadequate to initiate swimming if either cell was active alone (Fig. 9B). The coactivation of these two types of swim initiator cells, therefore, results in a summation of their effects on the swim pattern generator (Fig. 11).

In semi-intact preparations, intracellular stimulation of either cell 21 or 61 produced characteristic swimming movements of the leech body while bursts of neuronal activity, phase-locked to the swimming behaviour, were recorded from exposed segmental ganglia. In either semi-intact or isolated nerve cord preparations, cell 21- or cell 61-evoked swimming resembled swims initiated by segmental nerve

shock (Kristan & Calabrese, 1976), intracellular stimulation of cell 204 (Weeks & Kristan, 1978) or application of $>10^{-4}$ mol l^{-1} serotonin to isolated *Hirudo* nerve cords (Nusbaum, 1984). The similarities included (1) the latency to swim initiation (within seconds), (2) swim cycle period (0·5–2 s), (3) impulse burst duration and (4) impulse frequency of swim interneurones and motor neurones.

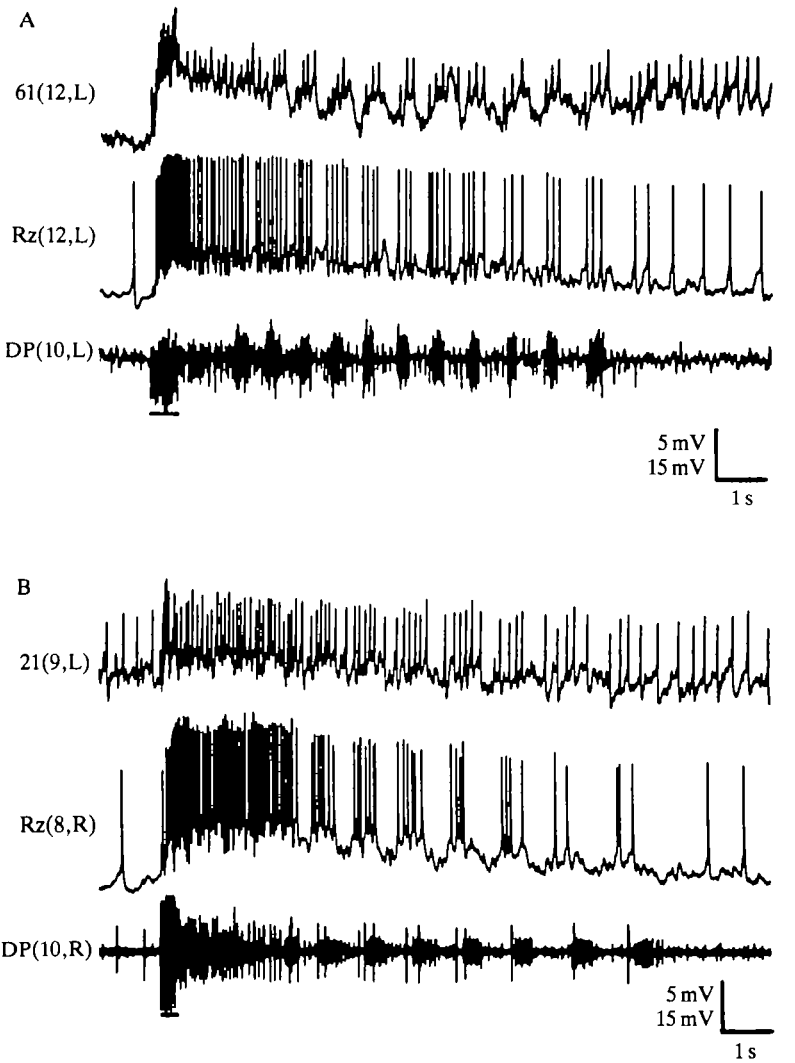

Fig. 8. Activity of the serotonin-containing cells during segmental nerve shock and the ensuing episode of the swim motor pattern. (A) Intracellular recordings of cell 61(12,L) and the Retzius cell (Rz) (12,L), with an extracellular recording from the dorsal branch of a posterior segmental nerve, DP(10,L) wherein occur repeating impulse bursts of an identified dorsal excitor motor neurone (cell 3) during swimming. (B) Intracellular recordings of cell 21(9,L) and the Retzius cell (8,R) with an extracellular recording from DP(10,R). In each case, the episode of swimming was initiated by a brief train of electrical pulses to DP(14) (not shown) for the time indicated by the bar at the bottom of the DP(10) recording. Preparation was a *Hirudo medicinalis* isolated nerve cord.

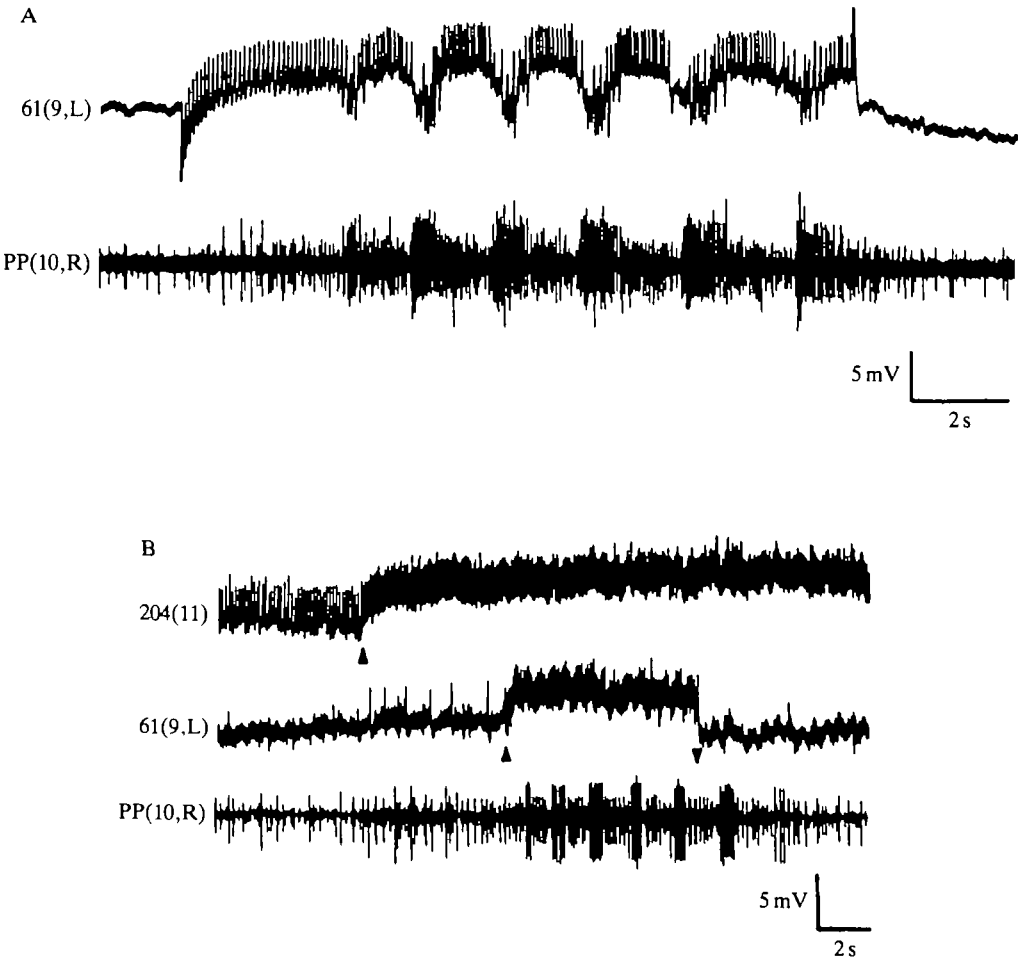

Fig. 9. (A) Swim initiation by intracellular stimulation of cell 61. Cell 61(9,L) was injected with 0·5 nA of depolarizing current for the duration of impulse activity in cell 61. The impulse bursts of the largest unit in the posterior branch of a posterior segmental nerve, PP(10,R) are those of a ventral excitor motor neurone. (B) Swim initiation by coactivation of cells 61 and 204. Paired intracellular recording of cell 204(11) and cell 61(9,L), with an extracellular recording of PP(10,R). Intracellular stimulation of cells 204 and 61 begins at the upward pointing arrowheads; termination of cell 61 stimulation at the downward pointing arrowhead. In this preparation, similar intracellular stimulation of cell 61(9,L) without stimulation of cell 204(11) did not initiate swimming (not shown).

In some cases, stimulating a single cell 21 or cell 61 in *M. decora* caused the swim motor pattern to be expressed locally, in only a few ganglia of a longer chain (Kristan & Nusbaum, 1983). In the same preparations, stimulation of cell 204 either immediately before or after cell 61 stimulation caused swimming to occur in the entire nerve cord. The localized 'swims' often had a much longer cycle period (2–4 s) than did swims involving the entire nerve cord. However, the latency to onset of a local 'swim' was not necessarily longer than that for swims involving the entire chain of ganglia. Similarly, in some semi-intact preparations only the front half of the

preparation would repeatedly undulate when cell 61 was stimulated, whereas both halves swam when cell 204 was stimulated. No local 'swims' were observed in response to stimulation of cells 21 or 61 in *Hirudo*.

Interactions of cells 61 and 204

The interactions of cells 61 and 204 were examined to determine the cause of their summating effects on swim-initiation. Depolarizing and hyperpolarizing current injected into either of these cells caused a similar but smaller amplitude change in both impulse activity and membrane potential in the other cell (Fig. 10). The strength of this interaction was so weak (similar to that between cell 61 and the other homoganglionic serotonin-containing cells) that the additive effects of cells 61 and 204 on swim-initiation probably result from their independent contact with the swim CPG (Fig. 11) (see also Nusbaum, 1986). There was also a polysynaptic excitatory effect of cells 61 and 204 upon each other *via* the swim central pattern generator. The following interactions between the serotonin-containing cells and cell 204 were also observed: (1) the interactions of cell 204 with the Retzius cell were similar to those with cell 61 (Fig. 10); (2) the electrical synaptic interactions of both cell 61 and the Retzius cell with cell 204 persisted under conditions where neurotransmitter release was depressed by elevated levels of Mg^{2+} ($25\,mmol\,l^{-1}$) in the saline (Fig. 10B); (3) cells 61 (or 21) and 204 also excited one another interganglionically, but the directness of this interaction was not tested. The intraganglionic interactions of cell 204 and cell 21 were not examined. Interactions of cell 21, cell 61 and the Retzius cell with cell 204 were not examined in *Hirudo*.

DISCUSSION

The two pairs of laterally-located serotonin-containing cells, cells 21 and 61, are both interganglionic interneurones which play a role in the initiation and maintenance of swimming behaviour in the leeches *M. decora* and *H. medicinalis*. Thus far, the only differences observed between these cells is that each cell has a unique morphology and cell body position. Possibly, with further study, physiological distinctions between these two serotonin-containing cells will become apparent.

Electrical coupling

Electrical synaptic interactions exist between all of the serotonin cells within a segmental ganglion (Lent & Frazier, 1977; Figs 4, 5). The pair of Retzius cells within each ganglion is strongly electrically coupled (the coupling coefficient being approximately 0·4) (Hagiwara & Morita, 1962). In contrast, the data presented in this paper indicate that the electrical coupling between a cell 21 or 61 and any other intraganglionic cell 21, 61 or Retzius cell in *Macrobdella* and *Hirudo* is sufficiently weak that each cell 21 and 61 may well be functionally independent in these species. Whether this difference from the study in *H. marmorata* (Lent & Frazier, 1977) is a species difference, or is due to the use of Neutral Red in the earlier study is unknown. The extent to which each of these serotonin-containing cells function separately or in

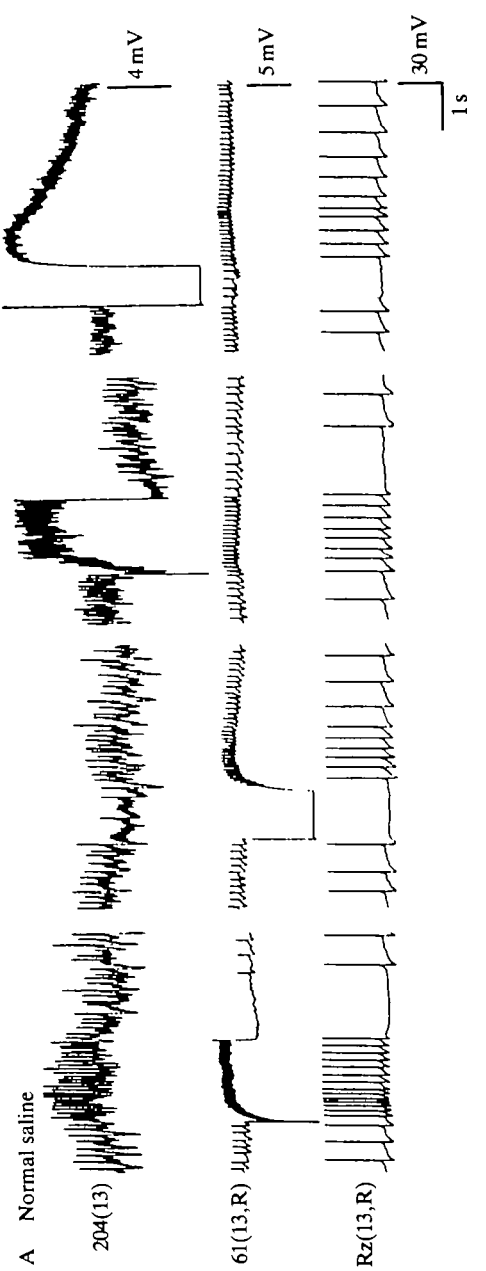

Fig. 10. Electrical coupling among cells 61, 204 and the Retzius cell (Rz) in the same ganglion. All recordings are intracellular. (A) Recordings made in normal saline. Injection of depolarizing (+1·0 nA) and hyperpolarizing (−5·0 nA) current into cell 61 caused an increase and decrease, respectively, in the firing frequency of cell 204 and the Retzius cell. Similarly, injection of depolarizing (+1·0 nA) and hyperpolarizing (−5·0 nA) current into cell 204 caused an increase and decrease, respectively, in the firing frequency of cell 61 and the Retzius cell. (B) Recordings made in 25 mmol l^{-1} Mg^{2+} saline. Higher levels of depolarizing current injection into cell 204 were required to observe a depolarization of the Retzius cell (not shown). Levels of current injection as in (A).

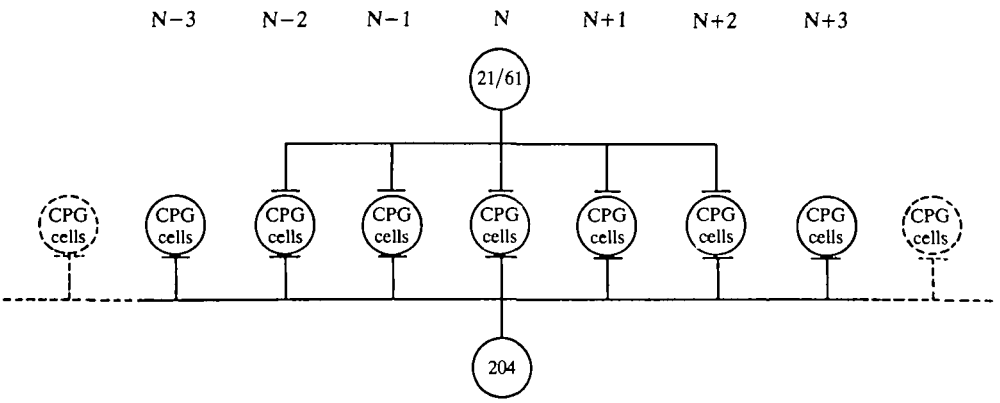

Fig. 11. Schematic summary diagram of the intersegmental extent to which cells 21/61 and cell 204 can directly excite the segmentally iterated swim central pattern generator (CPG). 'T' junctions indicate chemical synaptic excitation; segmental designations as in Fig. 5.

concert during a particular period, then, depends on their receiving common input. Common inputs, however, could also differentially affect the different serotonin-containing cells, as is the case for a pair of electrically coupled neurones in the lobster stomatogastric ganglion (Marder & Eisen, 1984). It is also possible that the strength of the electrical coupling among these cells could be modulated by other, as yet unidentified, inputs as is the case in the turtle retina (Piccolino, Neyton & Gerschenfeld, 1984).

The unusually long latency of the apparently electrical EPSP in the Retzius cell caused by cell 61 is not unique within the leech CNS (Friesen, 1985). One possibility that would account for such a delay is that the spike initiating zone of cell 61 may be closer to the cell 61 soma than is the site of electrical synaptic contact with the Retzius cell. This would cause a delay from the initiation of an impulse in cell 61 to its arrival at the junction with the Retzius cell that would depend on the impulse conduction velocity, whereas there would be a nearly instantaneous, passive propagation of the impulse from the point of its initiation towards the soma of cell 61.

Response to serotonin

Because the response of cells 21 and 61 and the Retzius cells to exogenously applied serotonin is inhibitory, direct activation of one serotonin-containing cell by the release of serotonin from another is unlikely. It is likely, however, that the serotonin cells excite each other by means of polysynaptic pathways, in addition to the weak excitation resulting from electrical coupling. One such polysynaptic excitatory pathway is *via* the swim central pattern generator, which is activated by each of the swim-initiator neurones and feeds back phasic excitation to these cells (Kristan & Weeks, 1983; Kristan & Nusbaum, 1983; Nusbaum, 1986).

Similar to its effect on leech serotonin-containing cells, serotonin also causes a direct hyperpolarization of serotonin-containing cells of the mammalian dorsal raphe

nucleus (Aghajanian & Lakoski, 1984). The receptors for serotonin on the terminals of these cells (autoreceptors) may serve to modulate serotonin release (Aghajanian, 1981; Middlemiss, 1982) and may also be responsible for the characteristic slow firing frequency of serotonin-containing neurones in both vertebrates and invertebrates (Aghajanian, 1981; Trulson & Jacobs, 1981; Granzow & Rowell, 1981; Fig. 4). These hypotheses may be directly tested both in the intact leech ganglion as well as by culturing individual cells 21, 61 and Retzius cells (Henderson, 1983). Fuchs, Henderson & Nicholls (1982) have shown that impulse-mediated serotonin release is reduced when cultured Retzius cells are hyperpolarized by current injection. A serotonin-induced decrease in the amount of serotonin released could be a determining factor in the termination of an episode of swimming.

Mechanosensory input

The pathway by which swim initiating levels of T, P and N mechanosensory cell excitation (Kristan, McGirr & Simpson, 1982; Kristan, 1983; Debski & Friesen, 1985) reach the serotonin-containing cells is only partly known (Fig. 7F). This excitation is partly due to the excitation that these cells receive from the S cell, a multimodal interneurone. However, intracellular stimulation of the S cell does not initiate swimming (Weeks, 1982*a*). A neurone that may mediate the P cell excitation of the serotonin-containing cells is a newly identified cell designated BN (Friesen & Brodfuehrer, 1984). Cell BN excites cells 21 and 61, and brief intracellular stimulation of cell BN triggers the initiation of swimming (Brodfuehrer & Friesen, 1984).

Initiation of swimming

Despite the weak electrical coupling between the various cells that initiate swimming, these cells are not all cophasically active during swimming (Weeks & Kristan, 1978; Weeks, 1982*a*; Fig. 8). Weeks (1981) has shown that the phasic nature of the activity pattern of cell 204 is not relevant to its role in the initiation and maintenance of the swim. The weakly phasic activity pattern of the serotonin-containing cells may also be irrelevant to their role in initiation and maintenance of swimming. In support of this hypothesis are the following observations: (1) tonic stimulation of either cell 21, cell 61 (Fig. 9) or the Retzius cell (Willard, 1981) initiates swimming; (2) chronic bath application of serotonin onto isolated nerve cords of *Hirudo medicinalis* causes swimming (Willard, 1981) and (3) serotonin injection into the coelomic cavity causes an increase in the amount of time spent swimming in intact, normal leeches (Willard, 1981) and enables swimming to occur in leeches that are unable to swim as a result of the selective ablation of their serotonin cells (Glover, 1984). Activation of CPGs by the tonic activity of CPG-initiator cells or pathways is a common feature of many invertebrate and vertebrate systems (e.g. Granzow & Kater, 1977; Weeks & Kristan, 1978; Fredman & Jahan-Parwar, 1983; Nagy & Dickinson, 1983; McCrohan, 1984; Grillner, 1985).

Relatively weak activation of a single cell 21 or 61, which may not activate the other serotonin-containing cells *via* electrical coupling, can sometimes initiate a swimming

episode. Therefore, the activity in any one of these cells may be sufficient to initiate the swim motor pattern. All the evidence accumulated so far, however, supports the conclusion that all of the cells 21 and 61, as well as the Retzius cells, normally contribute to swim initiation. For example, in order to initiate swimming, cell 21 or cell 61 usually must be stimulated to firing levels that also weakly activate the other serotonin-containing cells both within its ganglion and in each of the nearby anterior and posterior ganglia. The cumulative activity of these other cells would contribute significantly to the ensuing swim initiation. Additionally, each of the methods by which swimming is initiated (i.e. segmental nerve shock, mechanosensory stimulation or stimulation of cell 204 or cell 205) also activates cells 21 and 61 and the Retzius cells in most segmental ganglia.

Localized swims

At times, stimulating cell 21 or 61 causes only a subset of ganglia, within a longer chain of ganglia, to produce the swim motor pattern. Such localized 'swims' may occur because the restricted interganglionic axon of each cell 21 and 61 gives each of these cells a limited access to the segmentally iterated swim CPG (Fig. 11). Another swim-initiator neurone, cell 205, also elicits either localized or whole-cord swims from isolated *Hirudo* and *Macrobdella* nerve cords (Weeks, 1982*a*). Cell 205 has an interganglionic axon which contacts nine anterior segmental ganglia. In contrast to cells 21, 61 and 205, cell 204 has an interganglionic axon that extends over at least 14 ganglia and normally this cell exclusively causes whole-cord swims (Weeks, 1981). Severing one of its two interganglionic axons, however, sometimes causes cell 204 to produce swimming in only that part of the nerve cord still contacted by the intact axon (Weeks, 1980). It seems that although it is not necessary for the stimulated swim-initiator cell to contact the swim CPG in all ganglia to elicit a whole-cord swim, those ganglia that are so contacted are more likely to participate in the swim episode (Weeks, 1981, 1982*a*).

The ability of cells 21 and 61 to produce local 'swims' also indicates that they may be able to activate the swim CPG independently of cell 204 (see Nusbaum, 1986). What determines whether cell 21 or cell 61 initiates a local or cord-wide generation of the swim motor programme may be the extent to which it polysynaptically activates cell 204 in nearby ganglia. A localized 'swim' presumably occurs because feedback excitation from the swim CPG to cell 204 is inadequate to stimulate effectively a large number of cells 204, which otherwise would initiate swimming along the entire nerve cord.

The ability of the leech nerve cord to produce a localized swim may reflect the neuronal basis of the leech ventilatory behaviour. Ventilation is similar to swimming in that it involves repeated front-to-rear progressions of alternating dorsal and ventral undulations of the body, but it differs from swimming in that it (1) usually occurs while the rear sucker is attached to a substrate, (2) can occur at cycle periods inclusive of, but also considerably slower than those characteristic of swimming and (3) can occur in a restricted region of the leech, involving just a few body segments (Mann, 1962; W. B. Kristan & M. P. Nusbaum, unpublished observations). Given

the similarities between swimming and ventilation, it is likely that many of the components of the swim circuit also are used to mediate the ventilatory behaviour.

Comparison of swim-initiation by cells 21 and 61 with other leech swim-initiating neurones

Multiple central neurones capable of initiating the same central motor programme have been identified in a variety of animals (McClellan, 1983; Fredman & Jahan-Parwar, 1983; McCrohan, 1984; Marder, Hooper & Eisen, 1985). However, as yet little is known regarding the interactive effects of these different inputs on CPG activation. There are now three different identified cell types that can initiate leech swimming: cells 204 and 205, cells 21 and 61; and the Retzius cells. These cells all receive mechanosensory excitation that leads to swim-initiation (Weeks & Kristan, 1978; Willard, 1981; Weeks, 1982*a*; Fig. 7), as well as excitation from a higher-order interneurone that can trigger swim-initiation (Brodfuehrer & Friesen, 1984).

Initiation of swimming by cells 21 and 61 is similar to swim-initiation caused by cells 204/205 and differs from swim-initiation by the Retzius cell in two major respects. (1) The latency to swim onset is typically one to two orders of magnitude shorter following stimulation of cells 21, 61, 204 or 205 than it is following Retzius cell stimulation (Kristan & Nusbaum, 1983). (2) Only a single episode of swimming results from single stimulus trains to each of these swim-initiator cells except the Retzius cell, whose stimulation causes repeated episodes of swimming to occur for many minutes, suggesting a long-term humoral effect of the Retzius cell (Willard, 1981). In contrast to the humoral effect of the Retzius cell, cells 21 and 61 make discrete synaptic contacts with cells of the swim CPG (Kristan & Nusbaum, 1983; Nusbaum, 1984, 1986), as do cells 204 and 205 (Weeks, 1982*a,b*; Nusbaum, 1984). Additionally, cells 21 and 61 appear to act in parallel with cells 204 and 205 to effect swimming (Fig. 11).

Cells 204 and 205 plus the serotonin-containing neurones may constitute a command system for swim initiation. Although more effective at initiating swimming than are the individual serotonin-containing neurones, cells 204 and 205 lose their ability to initiate swimming when all of the serotonin-containing neurones are selectively ablated (Glover & Kramer, 1982; Glover, 1984). Swimming behaviour can then be restored temporarily by injecting serotonin into the coelomic cavity. Although not able to swim, these leeches do exhibit some of the behavioural components associated with preparing to initiate swimming, and the resulting behaviour includes some swim-like components (Glover & Kramer, 1982; Glover, 1984). Therefore, in the absence of serotonin, cells 204 and 205 are apparently still sufficient for initiating certain aspects of swimming. In the intact leech, the initiation of swimming is probably the result of contributions of all of the swim-initiating neurones. Retzius cells lower the threshold for swim initiation, and cells 21 and 61 act in concert with cells 204 and 205 to initiate swimming rapidly.

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