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POSITIONAL DISCRIMINATION AND RE-DEVELOPMENT OF SYNAPSES IN THE LEECH *WHITMANIA PIGRA*

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Summary

Identified neurones in the leech *Whitmania pigra* have a stable morphology with bilaterally symmetrical branching arborizations, and with axons on both sides arranged symmetrically in the connectives. Each anterior pagoda cell (AP) receives electrical and/or chemical synaptic input from mechanoreceptive cells on both sides of the body. The position in the body can be discriminated by the postsynaptic responses of the APs: as a rule, the responses to input from contralateral receptive neurones are stronger than those to input from ipsilateral ones, and the neurone with its receptive field on the dorsal side produces a stronger response than the neurone with a ventrally sited receptive field. APs integrate postsynaptic potentials and spikes. There are no connections between the two AP cells and so it is possible that positional discrimination depends upon a circuit comparing the inputs.

After the body wall has been cut round and rotated by 180°, the mechanoreceptive cells and annular erector motoneurones reinnervate the body wall strictly according to their original orientation, and repair is bilaterally synchronous. This eliminates a role for target cell guidance, particularly in the adult leech. When an extra Retzius cell is implanted into cultured ganglia, synapses develop between the host and the implanted neurone. Such synapses generally show lower coupling ratios or PSP fluctuations. However, the specific electrical connection between the Retzius cells shows a normal coupling ratio.

Introduction

The leech preparation used in this investigation has a number of advantages: (1) the sensory and motor neurones are identifiable and innervate defined receptive fields (Nicholls and Baylor, 1968) or muscle groups (Bowling *et al.* 1978); (2) in *Whitmania pigra* the axons of sensory and motor neurones have a stable structural pattern and show symmetrical arborizations in the ganglia and connectives; (3) a pair of identified neurones, the anterior pagoda (AP) cells, can discriminate and integrate the PSPs evoked by mechanoreceptive signals from both sides of the body. These neurones express their ability for spatial discrimination both centrally

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and peripherally. Centrally, discrimination occurs at the level of the synapse, where the strength of excitation varies depending upon the position of the receptive field. The second site is located at the growth cone of the peripheral terminal which differentiates the position to be innervated. The two problems, that of forming connections and that of signalling, involve very different mechanisms and different experimental approaches.

Expression of neurone morphology

The stability of neuronal architecture

The morphology of leech neurones was reviewed by Muller *et al.* (1981). Recently, the stability of axon architecture in embryonic leech has been recognized (Gao and Macagno, 1987). In order to assess the stability of axon architecture in *Whitmania pigra*, individual neurones were visualized by injection with horseradish peroxidase and the number of cells showing morphological variation was expressed as a percentage of the total number of neurones examined. In some cells no variation was found; 100 % of T cells (N=52), P cells (N=31) and N cells (N=177) had a stable morphology. Of the Retzius (R) cells, 88 % (71 of a sample of 81) showed a consistent axon pattern. The main variations of Retzius cell architecture occurred on the collateral branches towards the anterior connectives (Wang and Zhang, 1990). Of the 260 AP cells examined, 96 % had a stable morphology; from the second to the nineteenth segmental ganglion, the branching pattern was the same.

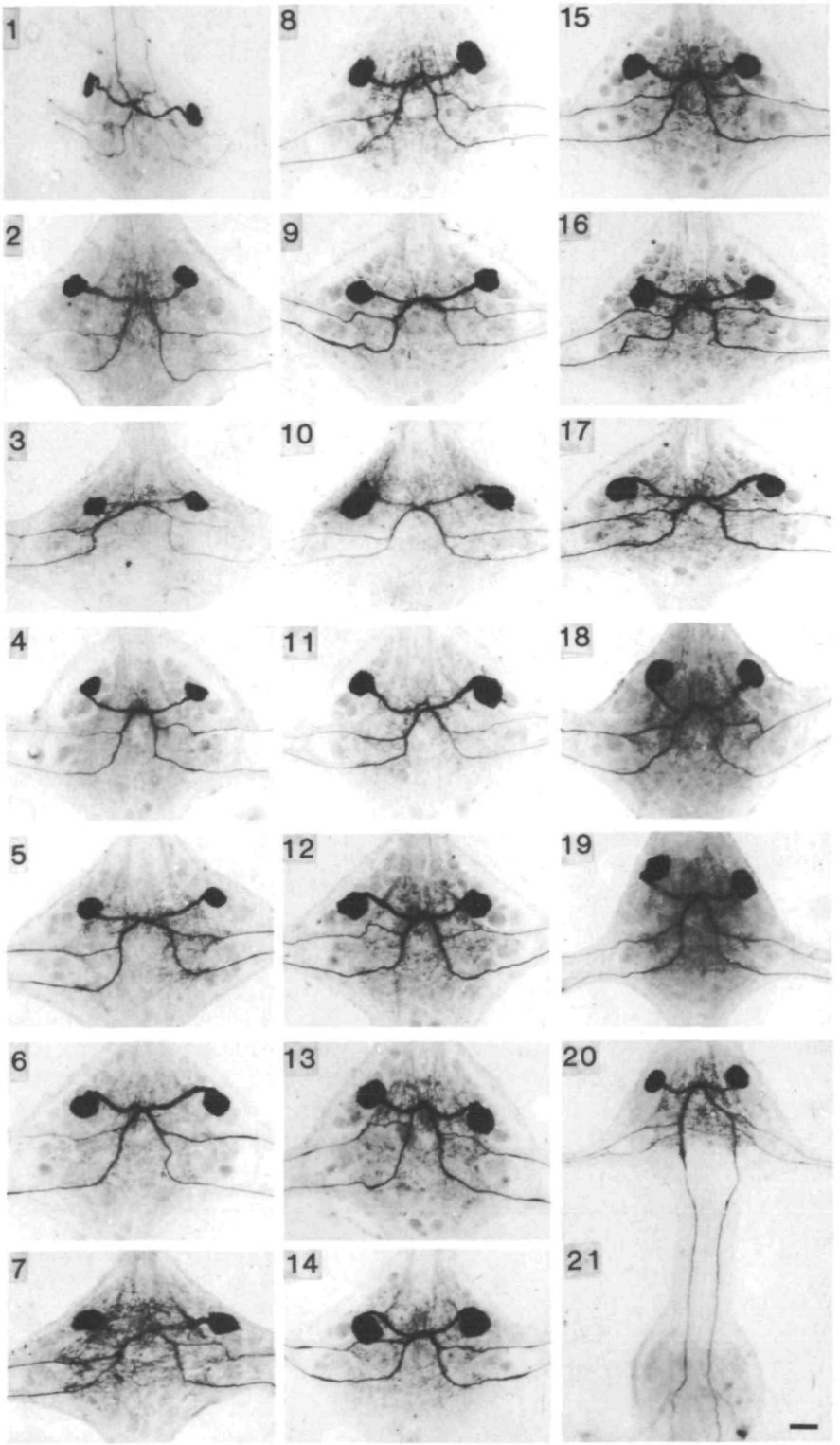
Symmetrical arborization of axons

In the leech the T, P and N mechanoreceptive cells supply receptive fields in the periphery. Similarly, the innervation of the annuli erector muscles on both sides of the body by the annuli erector neurones (AE) is symmetrical. Several neurones also show symmetrical axonal arborization within the mature central nervous system. In *Whitmania pigra*, T, P, N, AE and Retzius cells all show symmetrical axonal branching (Wang and Zhang, 1990) and AP neurones show symmetrical axonal branching throughout the chain of segmental ganglia (Fig. 1).

Symmetrical loci of axons in connectives

The central nervous system (CNS) of the leech consists of a series of ganglia linked by connectives. Neurones send out their axons to the adjacent anterior and posterior ganglia. Retzius cells send out a collateral axon from each of these branches towards the anterior and posterior roots separately, and also to the connectives. These branches are always located in a particular part of the connectives. Retzius and N cell axons are located in the ventral–lateral area of

Fig. 1. Symmetrical branching pattern of the AP neurones throughout the chain of segmental ganglia in *Whitmania pigra*. The order of the ganglia is indicated by the number. Scale bar, 50 μm.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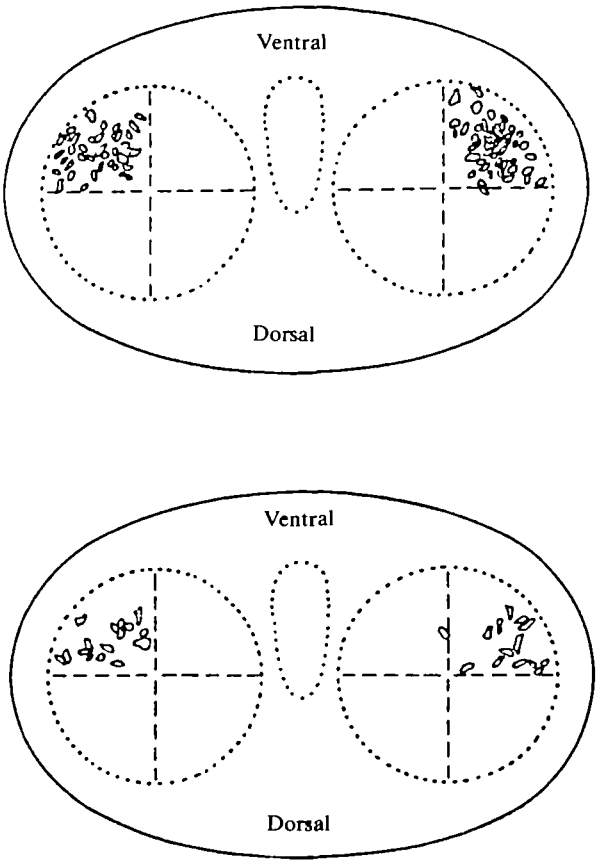


Fig. 2. Symmetrical loci of identified axons in connectives. Upper axon, loci of Retzius cells; lower axon, loci of N cells.

connectives (Fig. 2). There is no obvious difference in the position of these two neurones.

Positional discrimination and integration of receptive signals on AP neurones

Synaptic connections between receptive neurones and the AP cell

The AP neurone contains both acetylchonine (ACh) (Wallace, 1981) and FMRFamide (Kuhlman *et al.* 1988); its function is unknown, and its connections with other cells have not been ascertained. In *Whitmania pigra*, rectifying electrical synapses occur between the receptive cells (T, P, medial N) and the AP neurones. When ganglia were bathed in normal Ringer's solution no facilitation of excitatory postsynaptic potentials (EPSPs) could be found when paired electrical stimuli were delivered to medial P cells. When a depolarizing current was passed into a medial P cell bathed in a Ringer's solution containing 10 mmol l^{-1} Ca^{2+} or 15 mmol l^{-1} Mg^{2+}, EPSPs recorded in the contralateral AP neurone were the same as those recorded in normal Ringer. When a train of depolarizing impulses was

Table 1. *Types of synaptic connection between T, P and N sensory cells and the AP neurone*

	Electrical synapse	Chemical synapse
T	Rectifying	
P	Rectifying	
N		
Ipsilateral medial	Rectifying	
Contralateral medial	Rectifying	IPSP
Contralateral lateral		EPSP

passed to the medial P cell, synaptic facilitation and suppression were not seen on AP neurones; the coupling ratio recorded from a medial P cell when the hyperpolarizing current was passed into an AP neurone was only 0.01. This indicates that there is a rectifying connection between medial P and AP cells. The inhibitory chemical synapse from a medial N cell to an AP cell seems to be a polysynaptic connection. The connections between other cells and the AP neurone are shown in Table 1. In culture, the synapses formed between medial N and AP cells were either rectifying or non-rectifying electrical connections, but only rectifying connections formed between lateral N and AP cells (Vyklicky and Nicholls, 1988).

Positional discrimination of receptive fields by the AP neurone

To maintain a constant level of stimulus intensity from different sensory cells, depolarizing currents were passed into the somata of homologous dorsal, lateral and ventral T cells so that three spikes were produced. These spikes elicited EPSPs on the AP neurone showing obvious differences in amplitude. Thus, we can place in order of rank the strength of the excitation evoked by T cells with different receptive fields. In the three modalities of the mechanoreceptive cell, the rules are: (1) that the EPSPs evoked by the contralateral receptive cell are stronger than those evoked by the ipsilateral receptive cell; and (2) that the EPSPs elicited by the neurone with a dorsally situated receptive field (dorsal T cell) are stronger than those from the neurone with a ventrally situated receptive field (ventral T cell), while the neurone with a lateral receptive field (lateral T cell) produces EPSPs in the middle of the range.

Integration of signals from different receptor cells by the AP neurone

The AP neurone can sum the EPSPs elicited by the bilateral medial P cells from both sides (Fig. 3). The contralateral medial N cell elicits either an electrically induced EPSP or a chemically induced IPSP on the AP neurone, but the ipsilateral medial N cell produces only an electrically induced EPSP. The passage of a train of impulses simultaneously into the bilateral medial N cells resulted in the suppression of the spontaneous potentials of the AP neurone. The contralateral lateral N cell excited the AP cell to discharge action potentials. When a train of

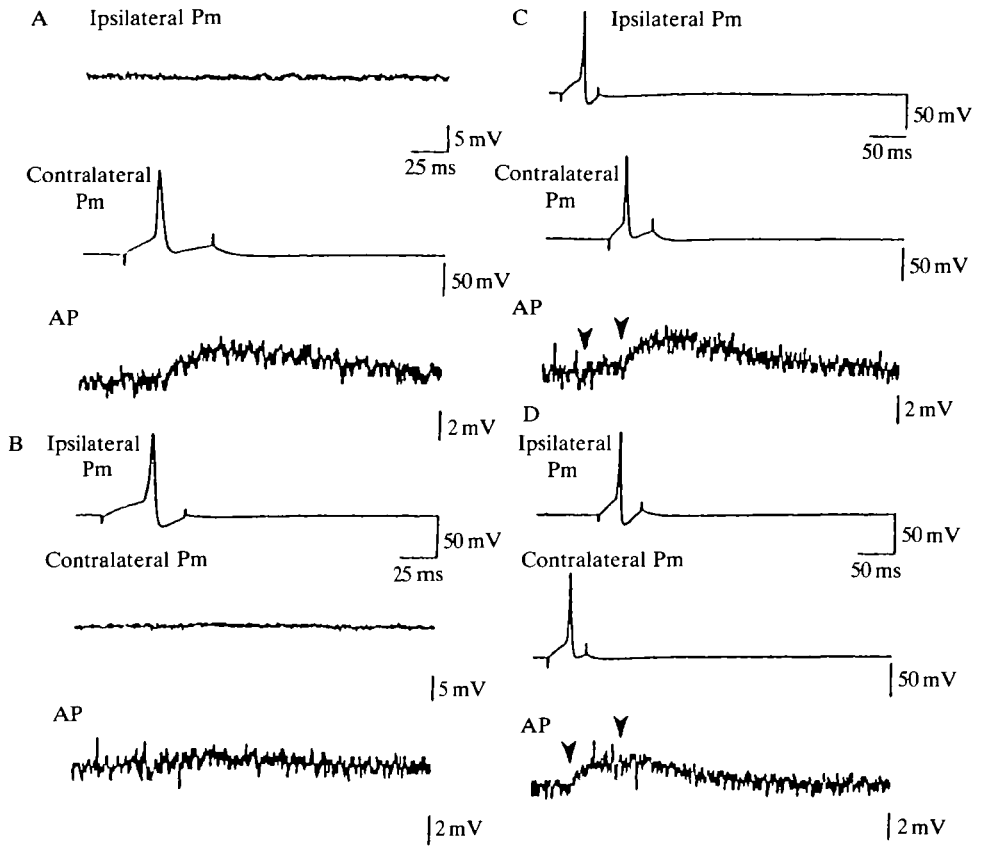
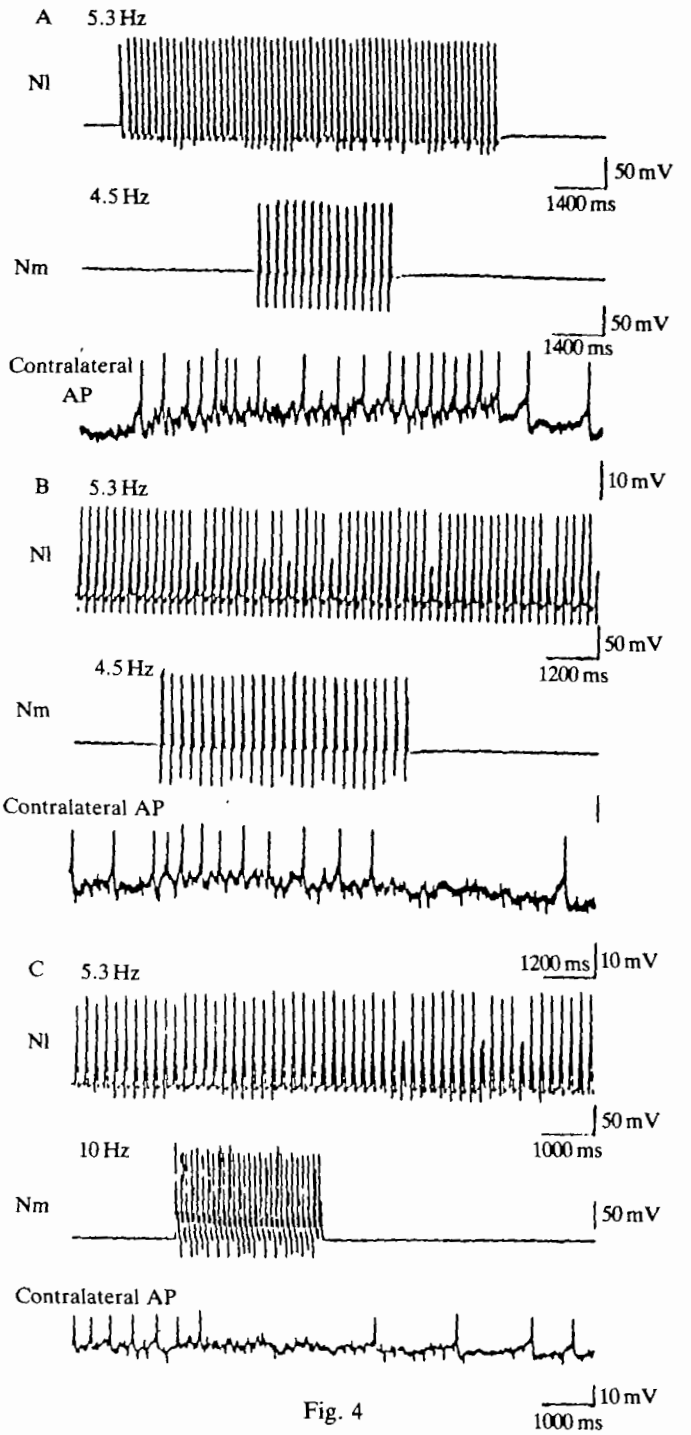


Fig. 3. Summation of EPSPs elicited by the bilateral medial P cells (Pm) on the AP neurone. The EPSP elicited by the contralateral Pm cell (A), and the ipsilateral Pm cell (B) acting independently on the same AP neurone; summation of EPSPs elicited by successive excitation of the Pm cells on both sides (C,D). Arrowhead, starting point of EPSP produced by each Pm cell.

current impulses was applied simultaneously to the contralateral lateral and medial N cells, the frequency of impulse discharge from the lateral N cell was decreased because of the suppression exerted by the medial N cell. An increase in the frequency of the current passing through the medial N cell enhanced the suppression effect upon the lateral N cell (Fig. 4).

The peripheral innervation of the AP neurone is not fully known, so we can only comment on its central function. The arborization of the AP neurone is morphologically similar to that of the L and the AE motor neurones, with

Fig. 4. Integration of EPSPs on an AP neurone. Passing current at 5.3 Hz into the contralateral N1 cell excites the AP cell; this is suppressed by passing a current at 4.5 Hz into the medial N cell on the same side (A); increasing the duration of the 4.5 Hz current passing into the medial N cell enhanced the suppression (B), as did increasing the frequency of the current to 10 Hz (C).


A
5.3 Hz
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50 mV
1400 ms
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Nm
50 mV
1400 ms
Contralateral AP
B
5.3 Hz
10 mV
Nl
50 mV
1200 ms
4.5 Hz
Nm
Contralateral AP
C
1200 ms
10 mV
5.3 Hz
Nl
50 mV
1000 ms
10 Hz
Nm
50 mV
Contralateral AP
10 mV
1000 ms

Fig. 4

contralateral innervation and with few synaptic varicosities. The axon branches of the AP cell can be seen in the anterior root, in the posterior root and in its dorsal branch, indicating that it is probably a motor neurone. The function of the AP neurone is similar to that of the L and AE neurone, since stimulating the T, P and N cells can elicit excitatory monosynaptic potentials in the ipsilateral and contralateral AP, L and AE neurones (Nicholls and Purves, 1970). However, for the AP cell, T and P cells elicit synergistic potentiation, while the medial N cell produces an antagonistic depression. In addition, the AP neurone can discriminate and integrate the afferent signals from the T, P and N receptive cells. There are no electrical or chemical connections linking the two AP cells bilaterally (Sunderland, 1980), but the L and AE cells do have such a connection.

Reinnervation of peripheral terminals

In a number of different animals changing or rotating the position of the target area has been used to study neural regeneration (Sperry, 1943; Miner, 1956; Jacobson and Backer, 1968; Bloom and Tompkins, 1976; Jacobson, 1978; Murphey, 1985; Koopowitz and Holman, 1988; Edwards, 1988). The results obtained from such experiments were variable. Nerves in the cricket or in *Xenopus* reinnervated their original target, but in the leech neurones kept their original spatial orientation after the body wall had been rotated in any one of three dimensions, even when the nerve roots had been crushed after rotation. This means that, in the course of reconstruction, regrowing nerves take the same pathway as they do in development and are uninfluenced by the position of the original target. Fig. 5 shows diagrammatically that the T cells reinnervate the original position of their receptive fields. This is also true of all the other

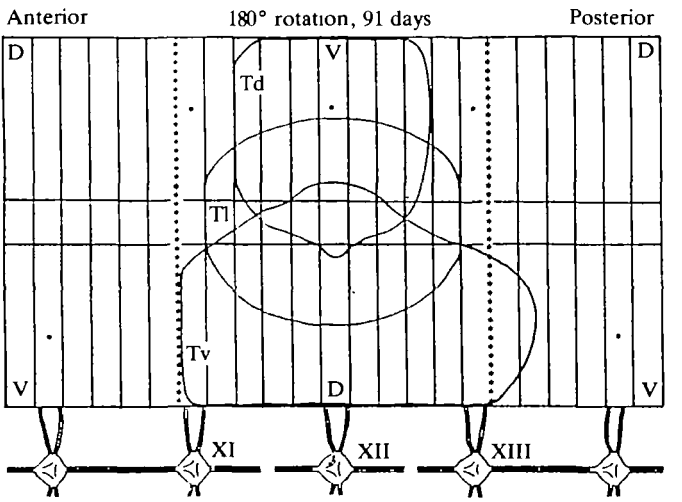

Fig. 5. Reconstruction of the receptive fields of the three T cells after the body wall had been rotated by 180° for 91 days. Td, dorsal T cell; Tl, lateral T cell; Tv, ventral T cell; ***, cut line; D, dorsal; V, ventral.

Table 2. *Reinnervated field after body wall rotation in* Whitmania pigra

	Number of annuli innervated			
	Receptive field			Contractive area
	T	P	N	AE
Normal leech	11	9	9	9
Body wall rotation				
360°, 150 days	7–9	7	9	
180°, 91–177 days	5–13	5–8	6–8	6–8
90°, 64 days	7–11	7	7	
Body wall rotated and root crushed				
90°, 45–47 days	10	6–7	7	9

AE, annular erector neurone.

mechanoreceptive cells and the AE motor neurone (Table 2). After the direction of the annulus erector (AE) muscles has been changed by 90°, the reinnervated muscles contract at right angles to the rest of the annuli. A more interesting phenomenon is that the three types of mechanoreceptive cells, T, P and N, are repaired synchronously. Similarly, the innervated areas of receptive and motor neurones on each side and the receptive and/or motor functions on each side are also repaired synchronously. Fig. 6 shows the bilateral reinnervation of a lateral P cell.

Formation of novel synapses in ganglia

The specificity of synaptic connections between identified neurones in the leech has been convincingly demonstrated *in situ* and also in culture (Nicholls, 1987). Recently, a further report has shown that when a single neurone is implanted into a cultured ganglion, novel synapses are formed and are characterized by features such as the fluctuation of postsynaptic potentials and a lower electrical coupling ratio (Zhang, 1989). In dissociated cell culture it is possible to induce the formation of electrical synapses; the electrical coupling ratio starts at a weak level, becoming stronger within 2 days (Liu and Nicholls, 1989). In contrast, the coupling ratio of synapses formed *in situ* is much higher. For example, in the ganglia, the electrical coupling ratio is not less than 0.5 when depolarizing current is passed into R–R cell pairs, and ranges from 0.5 to 0.9 for both original and novel connections formed by the third day. However, in dissociated cell culture, the coupling ratio ranges from 0.03 on the fourth day to 0.07 by day 21 (Table 3). An obvious difference between the *in situ* and the dissociated cell culture situation is the presence of glial cells *in situ*. The coupling ratio of glial cells is about 0.3 under hyperpolarizing current (Kuffler and Potter, 1964). After glial cells have been killed, the reformed S–S cell connection has a coupling ratio of 0.04 with

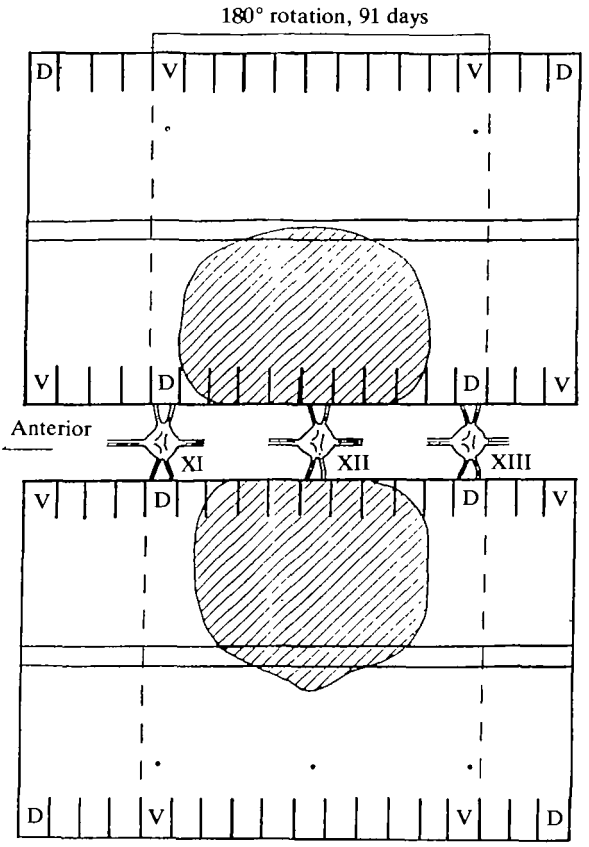

Fig. 6. Synchronous repair of bilateral Pl cells in the isolated ganglion XII 91 days after the body wall was rotated. Abbreviations as in Fig. 5.

depolarizing current and 0.06 with hyperpolarizing current (Elliott and Muller, 1983).

Electron microscope observations on gap junctions

Gap junctions between the terminals of homologous neurones

Electrical synaptic connections have been reported between many neurones in the leech; for example, between pairs of Retzius, T, L, AE and S cells (Muller *et al.* 1981). However, until now there have been few electron micrographs of these areas in the leech.

The gap junction between nerve terminals in the leech displays a gap of approximately 2 nm in width. Fig. 7 shows an electron micrograph of a putative gap junction, filled with lanthanum, between two terminals.

Neurone–glial gap junctions

The entire central nervous system is composed of two kinds of cell, neurones

Table 3. *Coupling ratios of electrical synapses between Retzius cells*

Paired cells		Coupling ratio DP	Coupling ratio HP	References
In normal ganglion				
R–R		0.8	0.2	Eckert (1963)
		0.8	0.3	Zhang (1987)
In cultured ganglion				
R–R		0.9	0.5	Miyazaki *et al.* (1976)
		0.6	0.2	Zhang (1989)
Implanted Retzius cell in cultured ganglion				
R′–R′	9 days	0.5	0.5	Zhang and Nicholls (1983)
R′–R	3 days	0.5	0.4	Zhang (1989)
R–R′	3 days	0.2	0.2	Zhang (1989)
Nl–R′	3 days	0.06		Zhang (1989)
L–R′	3 days	0.03		Zhang (1989)
R′–L	3 days	0.02		Zhang (1989)
Dissociated cell culture				
R–R	21 days	0.07	0.3	Fuchs *et al.* (1981)
	4 days	0.03	0.08	Liu and Nicholls (1989)
R–L	9 days	0.03	0.06	Fuchs *et al.* (1981)
R–Nm	7 days	0.008	0.03	Vyklicky and Nicholls (1988)
Nm–R	7 days	0.01	0.07	Vyklicky and Nicholls (1988)

R, Retzius cell *in situ*; R′, implanted extra Retzius cell; Nl, lateral N cell; Nm, medial N cell; L, longitudinal motor neurone. DP, depolarizing current; HP, hyperpolarizing current.

and glial cells, but 'what specialized structures or junctions (if any) occur between neurones and glia?' (Nicholls, 1981). Recently a few articles have appeared that suggest arthropod-type gap junctions occur between neurones and glia, e.g. in houseflies (Saint Marie and Carlson, 1985) and in crayfish (Cuadras and Marti-Subirana, 1987).

The leech CNS is a classical model for research on the functional relationship between neurones and glia (Kuffler and Potter, 1964; Kuffler and Nicholls, 1966). Its outstanding advantage is that there are only 10 glial cells in each ganglion and these have defined positions. The six capsules forming each ganglion are distributed symmetrically, and there is one glial cell in each capsule, two glial cells envelop the neuropile lying longitudinally along the midline and two gliocytes occur on each side of the ganglion, one in the anterior and one in the posterior connective. Thus, the anatomical distribution of glial cells is also symmetrical.

Conclusion

The central nervous system of the leech contains identified neurones that have symmetrical axonal arborizations. The anatomical distribution of glial cells is also symmetrical. This symmetry has been exploited by researchers for studying both

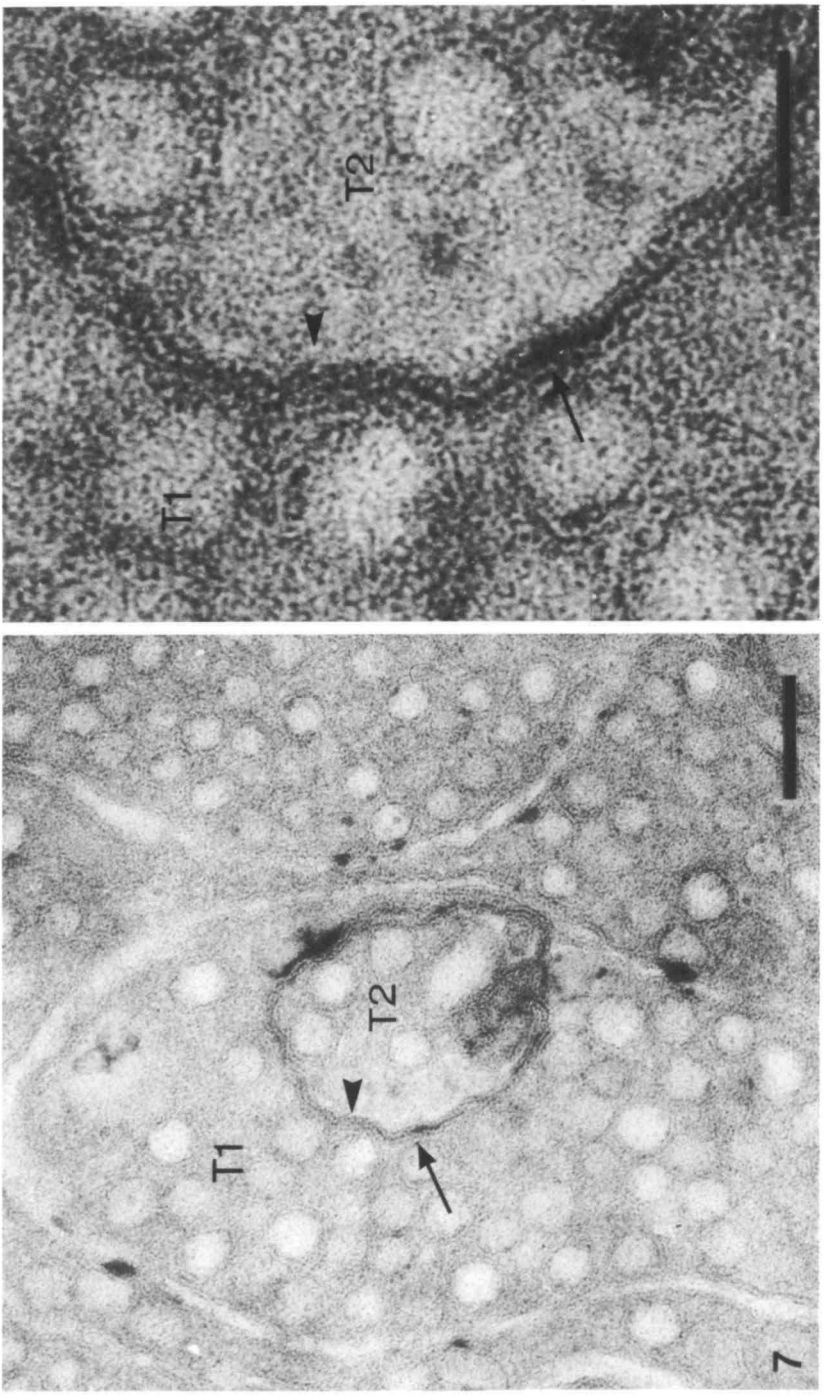

Fig. 7. (A) A neurone–neurone gap junction stained with lanthanum in *Whitmania pigra*. A gap junction between two terminals, T1 and T2. (B) An enlargement of A. Arrows point to the same gap junction; arrowheads to the same subunits. Scale bars, A 0.1 μm; B 0.05μm.

the integrative mechanisms for localization of sensory stimuli and the regeneration of synaptic connections in the ganglion. Afferent signals from the receptor neurones result in synaptic inputs that are discriminated and integrated by the AP neurone.

Our results give rise to a number of questions including those of whether position perception depends upon a neuronal circuit in the CNS comparing input from both sides of the body, and how the bilateral synchrony of neuronal repair is achieved. Is regeneration dependent upon the rate of axoplasmic transport and are glial cells involved in the process, for example by the production of extracellular attachment or other molecules? The central nervous system of leeches should provide an ideal preparation for answering such questions.

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References

BLOOM, E. M. AND TOMPKINS, R. (1976). Selective re-innervation in skin rotation grafts in *Rana pipien*. *J. exp. Biol.* **195**, 237–246.

BOWLING, D. J., NICHOLLS, J. G. AND PARNAS, I. (1978). Destruction of a single cell in the C.N.S. of leech as a means of analysing its connections and functional role. *J. Physiol., Lond.* **282**, 169–180.

CUADRAS, J. AND MARTI-SUBIRANA, A. (1987). Glial cells of the crayfish and their relationship with neurons. An ultrastructural study. *J. Physiol., Paris* **82**, 196–217.

ECKERT, R. (1963). Electrical interaction of paired ganglion cells in leech. *J. gen. Physiol.* **46**, 573–587.

EDWARDS, J. S. (1988). Sensory regeneration in arthropods: implications of homeostasis and of ectopic sensilla. *Am. Zool.* **28**, 1155–1164.

ELLIOTT, E. J. AND MULLER, K. J. (1983). Accurate regeneration of an electrical synapse between two leech neurons after destruction of the ensheathing glia cell. *J. Physiol., Lond.* **344**, 245–255.

FUCHS, P. A., NICHOLLS, J. G. AND READY, D. F. (1981). Membrane properties and selective connexions of identified leech neurones in culture. *J. Physiol., Lond.* **316**, 203–223.

GAO, W.-Q. AND MACAGNO, E. R. (1987). Extension and retraction of axonal projections by some developing neurons in the leech depend upon the existence of neighboring homologous. II. The AP and AE neurons. *J. Neurobiol.* **18**, 295–313.

JACOBSON, M. (1978). *Developmental Neurobiology*. 2nd edn. New York: Plenum Press.

JACOBSON, M. AND BACKER, R. E. (1968). Neuronal specification of cutaneous nerves through connections with skin grafts in the frog. *Science* **160**, 543–545.

KOOPOWITZ, H. AND HOLMAN, M. (1988). Neuronal repair and recovery of function in the polyclad flatworm, *Notoplana acticola*. *Am. Zool.* **28**, 1065–1075.

KUFFLER, S. W. AND NICHOLLS, J. G. (1966). The physiology of neuroglia cells. *Ergeb. Physiol.* **57**, 1–90.

KUFFLER, S. W. AND POTTER, D. D. (1964). Glia in the leech central nervous system: physiological properties and neuron–glia relationship. *J. Neurophysiol.* **27**, 290–320.

KUHLMAN, J. R., LI, C. AND CALABRESE, L. (1988). FMRFamide-like substance in the leech. I. Immunocytochemical localization. *J. Neurosci.* **5**, 2301–2309.

LIU, Y. AND NICHOLLS, J. G. (1989). Steps in the development of chemical and electrical synapses by pairs of identified leech neurons in culture. *Proc. R. Soc.* B **236**, 253–268.

MINER, N. (1956). Integumental specification of sensory fibers in the development of cutaneous local sign. *J. comp. Neurol.* **105**, 161–170.

MIYAZAKI, S., NICHOLLS, J. G. AND WALLACE, B. G. (1976). Modification and regeneration of

synaptic connections in culture leech ganglia. *Cold Spring Harbor Symp. quant. Biol.* **40**, 483–493.

MULLER, K. J., NICHOLLS, J. G. AND STENT, G. S. (1981). *Neurobiology of the Leech*. New York: Cold Spring Harbor Laboratory, pp. 35–40.

MURPHEY, R. K. (1985). Competition and chemoaffinity in insect sensory system. *Trends Neurosci.* **8**, 120–125.

NICHOLLS, J. G. (1981). Introduction. Perspectives on the cell biology of glia. In *Glial–Neuron Interaction* (ed. J. E. Treherne), pp. 3–5. Cambridge, London: Cambridge University Press.

NICHOLLS, J. G. (1987). The search for connection: *Studies of Regeneration in the Nervous System of the Leech*. Sunderland, MA: Sinaeur Associates Inc.

NICHOLLS, J. G. AND BAYLOR, D. A. (1968). Specific modalities and receptive fields of sensory neurons in the CNS of leech. *J. Neurophysiol.* **31**, 740–756.

NICHOLLS, J. G. AND PURVES, D. (1970). Monosynaptic chemical and electrical connexions between sensory and motor cells in the central nervous system of the leech. *J. Physiol., Lond.* **209**, 647–667.

SAINT MARIE, R. L. AND CARLSON, S. D. (1985). Interneuronal and glial–neuronal gap junctions in the lamina ganglionaris of the compound eye of the housefly, *Musca domestica*. *Cell Tissue Res.* **241**, 43–52.

SPERRY, R. W. (1943). Effect of 180 degree rotation of the retinal field on visuomotor coordination. *J. exp. Zool.* **92**, 263–279.

SUNDERLAND, A. J. (1980). A hitherto undocumented pair of neurons in the segmental ganglion of the leech which receive synaptic input from mechanosensory cells. *Comp. Biochem. Physiol.* **67**A, 299–302.

VYKLICKY, L. AND NICHOLLS, J. G. (1988). Specificity of connections formed by nociceptive cells of the leech in tissue culture. *J. exp. Biol.* **134**, 17–26.

WALLACE, B. G. (1981). Neurotransmitter chemistry. In *Neurobiology of the Leech* (ed. K. J. Muller, J. G. Nicholls and G. S. Stent), pp. 147–172. New York: Cold Spring Harbor Laboratory.

WANG, D.-B. AND ZHANG, R.-J. (1990). The morphological similarity and symmetry of neurons in the leech, *Whitmania pigra*. *Sci. in China (Series B)* **33**, 821–826. (In Chinese).

ZHANG, R.-J. (1987). Chinese leech *Whitmania pigra* as a suitable specimen for neurobiological research. *Chinese J. physiol. Sci.* **3**, 416–423.

ZHANG, R.-J. (1989). Re-development of synaptic connections after implanted single 5-HT containing neuron in isolated leech ganglia. *Sci. in China (Series B)* **32**, 88–95.

ZHANG, R.-J. AND NICHOLLS, J. G. (1983). Identified neurons implanted into leech ganglia sprout and form synaptic connection. *Devl Brain Res.* **8**, 131–135.