

ELECTROPHYSIOLOGICAL IDENTIFICATION  
OF NEURONES AND NEURAL NETWORKS IN THE  
PERIOESOPHAGEAL GANGLION COMPLEX OF  
THE MARINE PULMONATE MOLLUSC,  
*ONCHIDIUM VERRUCULATUM*

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(Received 21 May 1973)

INTRODUCTION

It is well known that there are identifiable giant neurones in the central nervous system of some invertebrates. Many research workers have reported on the identification of the neurones and neural circuits in *Aplysia* (Hughes & Tauc, 1963; Tauc & Hughes, 1963; Hughes & Chapple, 1967; Kandel *et al.* 1967; Kandel, Frazier & Wachtel, 1969; Frazier *et al.* 1967; Kupfermann & Kandel, 1969; Waziri, Kandel & Frazier, 1969; Waziri & Kandel, 1969; Gardner, 1971; Weevers, 1971), in *Tritonia* (Willows & Hoyle, 1968), in *Anisodoris* (Gorman & Mirolli, 1969, 1970), and in *Planorbis* (Berry, 1972). These investigations are very important from the viewpoint of system engineering, because the nervous system is assumed to be a huge information-processing system, and for clarifying the function of the nervous system we must know the properties of the elements of it and also the connexions between and among the elements.

In the central nervous system of the marine pulmonate mollusc, *Onchidium verruculatum*, about thirty giant neurones were found with diameters of 100-300  $\mu\text{m}$ . Their properties have been investigated in relation to the fluctuation of electrical excitability during a refractory period (Katayama, 1971). However, systematic identification of neurones and neural networks in this material was almost absent. Therefore, we have made electrophysiological observations on *Onchidium verruculatum* for the identification of neurones and neural connexions. Some results have already been described concerning input and output pathways of neurones from and to the periphery (Katayama, 1970). The present paper will describe firstly the identification of individual neurones and secondly the connexions between and among the identifiable neurones.

MATERIALS AND METHODS

The perioesophageal ganglia of the marine pulmonate mollusc, *Onchidium verruculatum*, were carefully isolated from the animal body under a dissecting microscope and were immersed in circulated sea water maintained at 15-17 °C throughout all experimental procedures. Under the microscope we could easily observe the somata of the giant neurones and thrust one or two glass-capillary microelectrodes into each

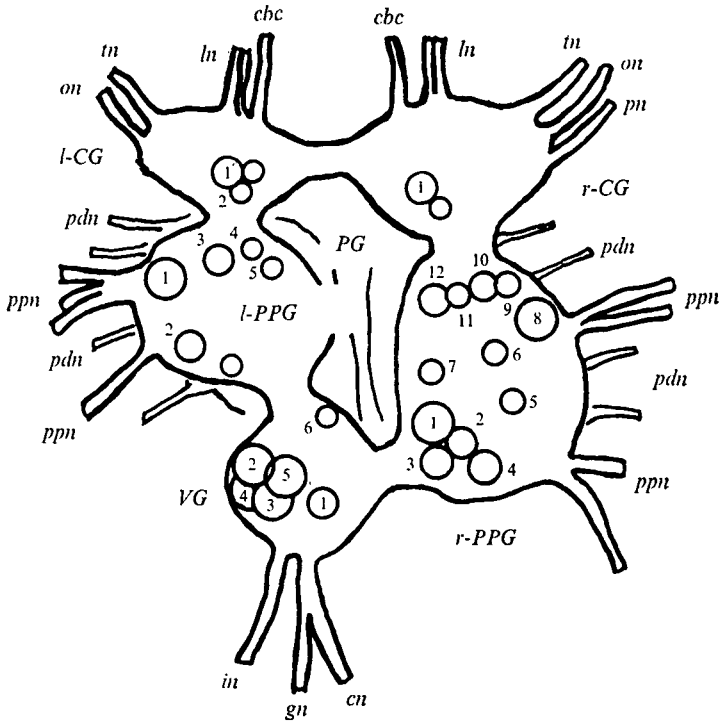


Fig. 1. Schematic illustration of the dorsal view of the perioesophageal ganglion complex of *Onchidium verruculatum*.

Anterior end is toward the upper of illustration. The designation of each ganglion is indicated; CG (cerebral ganglion), PPG (pleuroparietal ganglion), VG (visceral ganglion), and PG (pedal ganglion). The names of the peripheral nerve bundles and connectives are also indicated; *ln* (labial nerve), *on* (optical and olfactory nerve), *tn* (tentacular nerve), *pn* (penis nerve), *ppn* (pleuroparietal nerve), *pdn* (pedal nerve), *gn* (genital nerve), *in* (intestinal nerve), *cn* (cardiac nerve), and *cbc* (cerebro-buccal connective). Giant neurones visually identified are described at their loci and numbered for the sake of convenience.

of them for recording of membrane potential. The microelectrodes, filled with 2 M potassium citrate, had resistances of 20–40 megohms.

The fluctuation of membrane potential obtained by the intracellular microelectrode was amplified with a conventional D.C. amplifier of 40 dB gain, and was displayed on an oscilloscope or recorded on magnetic tapes. Technical details about experimental procedure have been reported elsewhere (Katayama, 1970, 1971).

## RESULTS

### *Visual observation*

The perioesophageal ganglion complex is composed of 7 ganglia: right and left cerebral ganglion (*r-CG* and *l-CG*), right and left pedal ganglion (*r-PG* and *l-PG*), right and left pleuroparietal ganglion (*r-PPG* and *l-PPG*), and visceral ganglion (*VG*). A dorsal view of them is schematically shown in Fig. 1. After white rough connective tissue covering the perioesophageal ganglia had been carefully removed, the somata of about 30 giant neurones were clearly visible under the dissecting microscope. Our

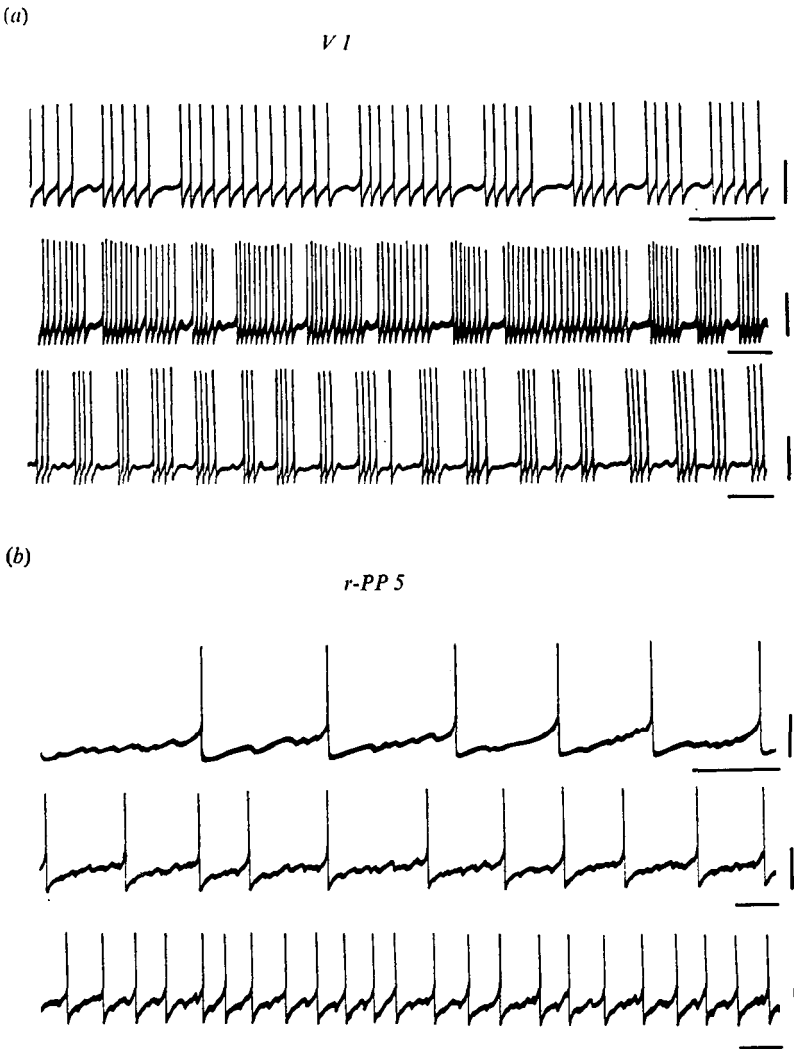


Fig. 2. Intracellular recordings of the giant neurones *V 1* and *r-PP 5* in (a) and (b), respectively. Time calibration, 1 sec; potential calibration, 10 mV, throughout all traces of this illustration. See text for details.

histological studies by optical microscope revealed the superficial disposition of the somata of the neurones surrounding a core of neuropile.

They were distinguishable from one another by the relative locations, sizes, and pigmentations of their somata. They were designated separately, for example *r-PP 1*. Each of them was designated by two symbols, one represented the abbreviated name of the ganglion to which it belonged and the other was a conveniently chosen numeral. The locations of the visually identified neurones are shown schematically in Fig. 1. On the basis of those findings further electrophysiological investigations were carried out under visual control.

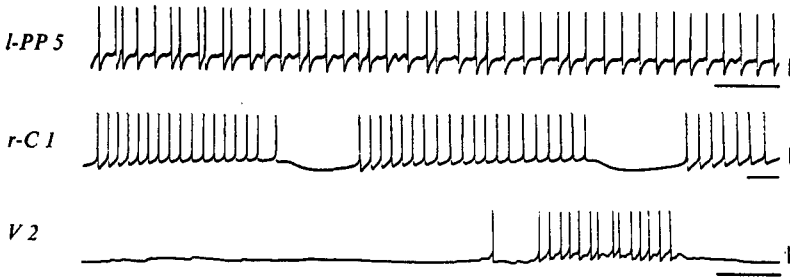


Fig. 3. Three examples of intracellular recordings of giant neurones *l-PP 5*, *r-C 1*, and *V 2*. Calibrations are 1 sec and 10 mV. The *l-PP 5* neurone received some EPSPs and several IPSPs, firing frequently. The *r-C 1* neurone showed burst activity without PSPs. The *V 2* neurone showed also burst activity responding to many arriving EPSPs.

Table 1. *Input-output pattern of visually identified giant neurones without artificial stimuli to the preparation*

Neurone	Inputs*	Output pattern	Size of soma†
<i>V 1</i>	None	Almost regular	Small
<i>V 2</i>	E (grouped) & I	Irregular or burst	Large
<i>V 3</i>	None	Regular (low freq.)	Medium
<i>V 4</i>	I	Irregular	Large
<i>V 5</i>	E, I	Irregular	Large
<i>V 6</i>	E, I	Irregular	Small
<i>l-PP 1</i>	E, I	Silent	Large
<i>l-PP 2</i>	I	Silent	Medium or small
<i>l-PP 3</i>	E, I	Silent	Small
<i>l-PP 4</i>	E, I	Irregular	Small
<i>l-PP 5</i>	E, I	Almost regular	Small
<i>l-C 1</i>	None	Regular	Medium
<i>l-C 2</i>	E, I	Irregular	Small
<i>r-PP 1</i>	E, I	Irregular	Large
<i>r-PP 2</i>	E, I	Irregular	Medium
<i>r-PP 3</i>	None	Silent	Medium
<i>r-PP 4</i>	E	Irregular	Large
<i>r-PP 5</i>	I (many)	Irregular	Medium
<i>r-PP 6</i>	E, I	Irregular	Medium
<i>r-PP 7</i>	I	Irregular (high freq.)	Medium
<i>r-PP 8</i>	E, I	Silent	Large
<i>r-PP 9</i>	E, I	Irregular	Small
<i>r-PP 10</i>	E, I	Silent	Small
<i>r-PP 11</i>	None	Regular	Small
<i>r-PP 12</i>	E, I	Irregular or burst	Small
<i>r-C 1</i>	None	Burst	Medium

\* E: EPSP; I: IPSP.

† The diameter of the soma was as follows: large, larger than about 300  $\mu\text{m}$ ; medium, between 200 and 300  $\mu\text{m}$ ; and small, smaller than 200  $\mu\text{m}$ .

#### *Input-output patterns of identifiable neurones*

The neurone *V 1* in Fig. 2*a* fired almost regularly, without apparent synaptic inputs, except for the silent intermissions of spike discharges. The slow waves during the intermissions were considered to be not IPSPs but aborted pre-potentials not attaining to the firing level, because the duration of the intermission was 2–4 times the shortest interval between spikes on each trace. This pattern was always observed in the *V 1* neurone in all preparations.

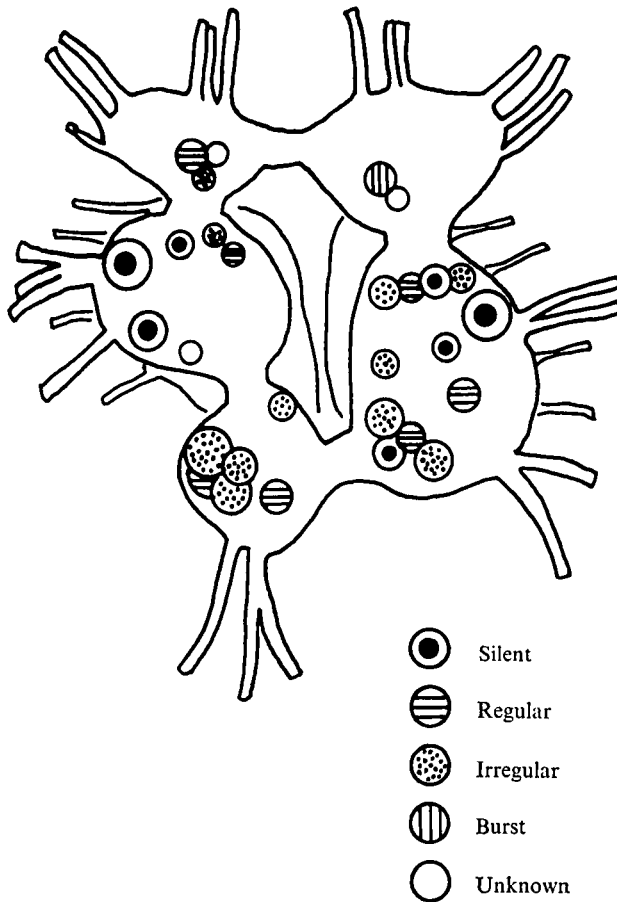


Fig. 4. Schematic illustration of distribution of visually identified giant neurones in Fig. 1, according to output discharge pattern summarized in Table 1.

Although the neurone *r-PP 5* showed numerous IPSPs and no EPSPs, it fired repetitively in all preparations, as shown in Fig. 2*b*. Interspike intervals of this neurone were dependent on the number of IPSPs; IPSPs were more frequent and the intervals were longer in the upper two traces than in the bottom trace.

Fig. 3 shows other examples of intracellular recordings. The neurone *l-PP 5* received some EPSPs and a few IPSPs, and fired frequently. The neurone *r-C 1* showed spontaneous grouped discharges without noticeable synaptic inputs. The neurone *V 2* also showed grouped discharges in response to tonic bombardment of excitatory synaptic inputs. Furthermore, the neurone *l-PP 1* was always silent and devoid of synaptic inputs. The input-output pattern was characteristic of each neurone, as summarized in Table 1, together with its morphological feature. Thus, visually identified neurones in Fig. 1 were electrophysiologically classified by their input-output pattern and described in Fig. 4, making it possible to study connexions between the individual neurones. But the significance of the location of the individual neurones was not established in regard to their functional roles.

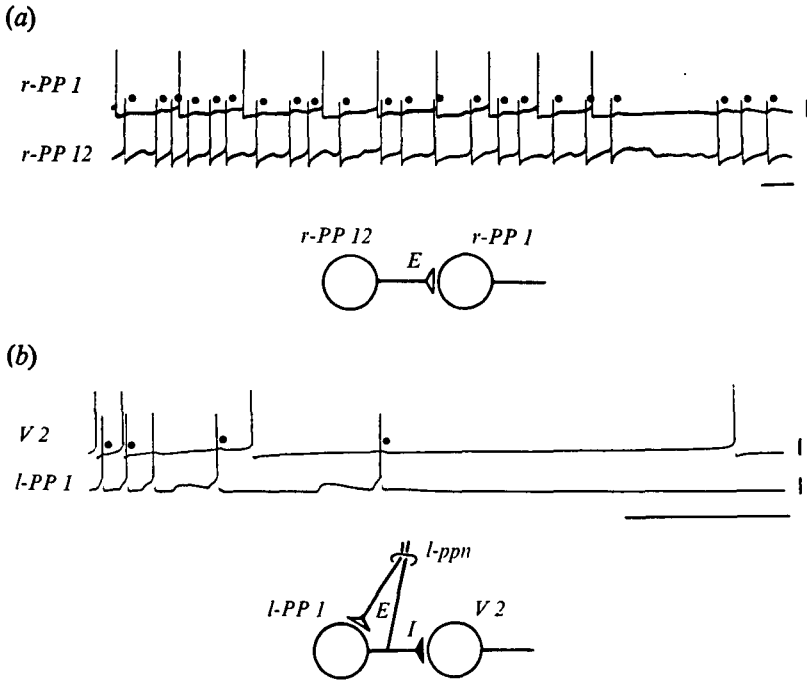


Fig. 5. Intracellular recordings of paired neurones connected directly. Calibrations: time, 1 sec and membrane potential, 10 mV.

(a) The spike discharges of the neurone *r-PP 12* are followed by EPSPs of the neurone *r-PP 1*, as indicated by filled circles. They are interconnected probably by an excitatory synapse (*E*), as shown in inset drawing.

(b) As indicated by the filled circles, IPSPs of the neurone, *V 2*, are observed after the spike of the neurone *l-PP 1*. Inset figure shows that they are connected by an inhibitory synapse (*I*). See text for the relation between the *l-PP 1* neurone and the *l-ppn* (left pleuroparietal nerve).

### *Connexions among and between identified neurones*

Simultaneous intracellular records of an arbitrarily chosen pair of neurones revealed how the paired neurones should be interconnected. Although about 30 neurones were visible on the dorsal surface of the periesophageal ganglia under the dissecting microscope, ten of them were excluded from further experiments because of their inaccessible locations and smaller sizes. We have examined 190 pairs among 20 neurones, i.e.  ${}_{20}C_2 = 190$ .

#### *(A) Direct excitatory and inhibitory connexions*

As shown in Fig. 5a, impulses of the neurone, *r-PP 12*, were followed by EPSPs of the neurone, *r-PP 1*, as indicated by filled circles. The axon of the former terminated on the latter, as illustrated by inset figure. One more excitatory direct connexion was found from the neurone *V 5* to one of unidentified neurones in the *r-PPG*.

As shown in Fig. 5b, IPSPs indicated by filled circles on the record of the neurone *V 2* were preceded by impulses of the neurone, *l-PP 1*, which was activated antidromically and orthodromically by a stimulus to the peripheral nerve bundle to body wall, *l-ppn* (see Fig. 1). The neurone *l-PP 1* might send its axon or axonal branches in the bundle *l-ppn* and receive excitatory synaptic inputs from the nerve bundle, and

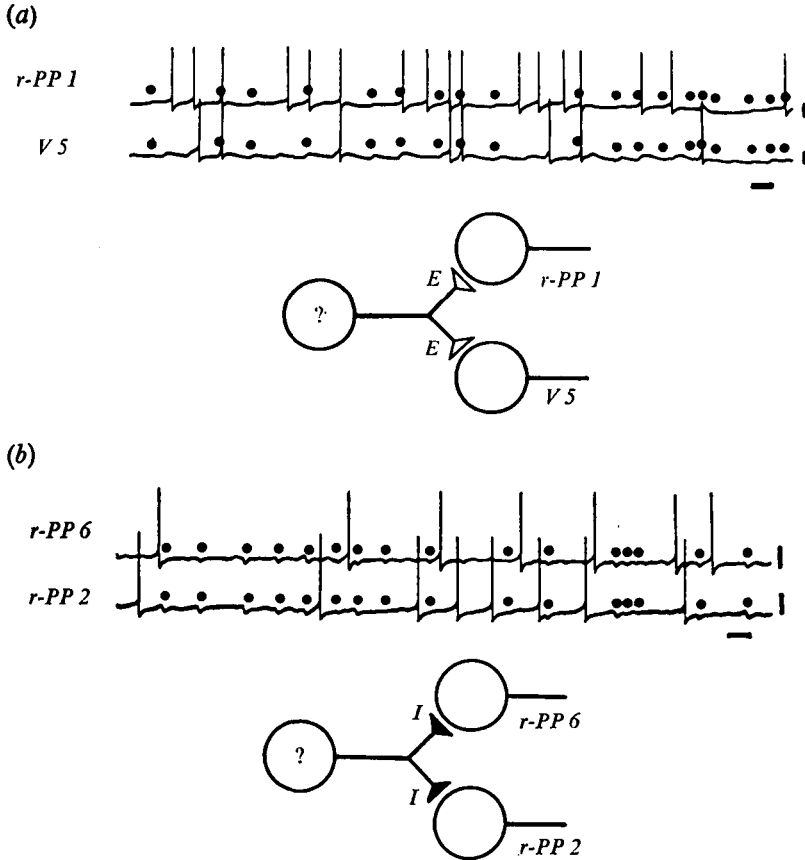


Fig. 6. Intracellular recordings of the paired neurones receiving synchronous PSPs. Calibrations: time, 1 sec and membrane potential, 10 mV.

(a) The neurones *r-PP 1* and *V 5* received synchronous EPSPs as indicated by the filled circles on both traces. They have a common source of EPSPs, as shown in the inset figure, i.e. an unidentified neurone indicated by a question mark may make contact with both of the neurones by excitatory synapses (*E*).

(b) The neurones *r-PP 2* and *r-PP 6* received synchronous IPSPs as indicated by filled circles on both of the records. Inset illustration shows that they might receive simultaneous inhibitory inputs through inhibitory synapse (*I*) from common source indicated by a question mark.

inhibit monosynaptically the neurone *V 2* as shown in the inset illustration of Fig. 5*b*. One more inhibitory direct connexion from the neurone *r-PP 1* to the neurone *r-PP 5* was observed.

Thus, two excitatory and inhibitory trans-synaptic connexions were detectable in our experiment.

### (B) Pairs of neurones with common input source

Synchronous EPSPs were observed on the records of the *r-PP 1* and *V 5* neurones, as shown in Fig. 6*a*. They were often evoked trans-synaptically by stimuli to the intestinal nerve (*in* in Fig. 1). An unidentified interneurone, activated by the peripheral stimuli, might produce the EPSPs in both neurones, as illustrated by inset figure. Simultaneous IPSPs were found on the records of the *r-PP 2* and *r-PP 6* neurones,

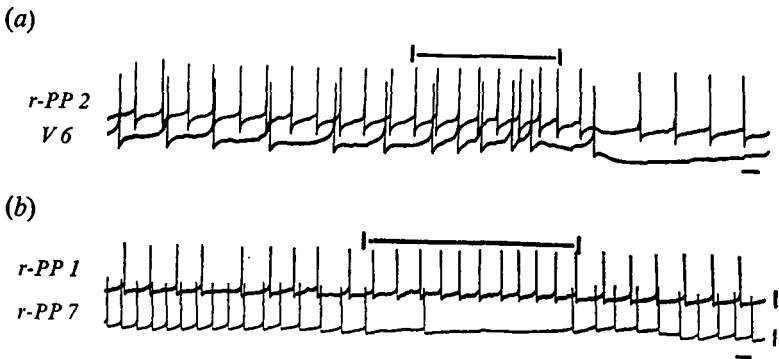


Fig. 7. Intracellular recordings of specified pairs of identified neurones. Calibrations: time, 1 sec and membrane potential, 10 mV.

(a) The neurones *r-PP 2* and *V 6* show a similar firing pattern; they elicited spikes frequently during the period indicated by the horizontal bar.

(b) The neurones *r-PP 1* and *r-PP 7* show an alternating firing pattern; during the period indicated by the horizontal bar, the one of them, e.g. *r-PP 1*, received many excitatory inputs and fired frequently, and the other, e.g. *r-PP 7*, was inactive, while during the other period the relation between them was reversed.

as shown in Fig. 6*b*. These neurones received inputs from the common input source not yet identified.

Synchronous EPSPs were observed in the pair of the neurones *l-C 2* and *V 5*, and IPSPs were also recorded in the pair of neurones *r-PP 2* and *r-PP 12*.

### (C) Pairs of neurones showing similar or alternative activity pattern

Both the *r-PP 2* and the *V 6* neurones in Fig. 7*a* received many EPSPs and fired frequently during the period indicated by a horizontal bar, showing a similar activity pattern. But the timings of the EPSPs and the spike discharges in both neurones were not synchronous. They are probably excited by and through a group of common interneurones. The neurones *r-PP 1* and *r-PP 2* also showed a similar activity pattern.

As shown in Fig. 7*b*, during the period indicated by a horizontal bar, the neurone *r-PP 1* received many EPSPs and fired frequently, while the other neurone *r-PP 7* was inactive. But for the other period their input and output patterns were reversed. Thus they showed an alternative activity pattern. They may be connected not directly but through several interneurones, as mentioned later in the Discussion.

### Neural network

In the preceding study we averaged the fluctuation of intracellular potential induced by the stimuli delivered to peripheral nerves connected with the perioesophageal ganglia, and successfully identified input and output pathways of the neurones in relation to the peripheral nerves (Katayama, 1970). The previous section of the present paper described the relationship between paired giant neurones. The knowledge, thus obtained, enabled us to construct neural networks.

Here we have focused in particular on one identified neurone, *r-PP 1*, as one of examples obtained in this series of studies, and the tentative neural network around the *r-PP 1* neurone is presented in Fig. 8. The neurone *r-PP 1* accepted trans-synaptic inputs from giant neurones in the ganglia and from the periphery directly or via inter-



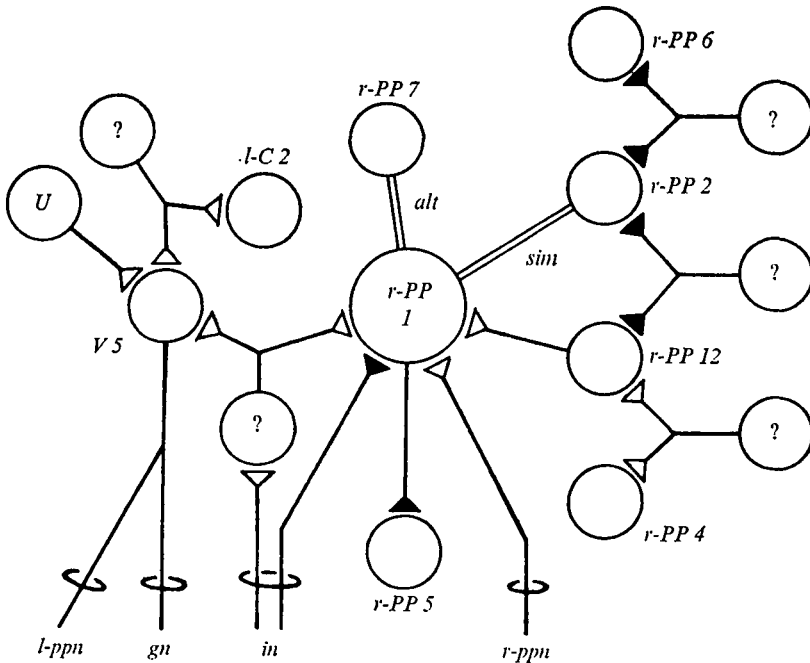


Fig. 8. Tentative neural network around the neurone *r-PP 1* in *r-PPG* (right pleuroparietal ganglion).  $\triangleleft$  and  $\blacktriangleleft$  represent the excitatory and the inhibitory connexions, respectively. The neurones indicated by question mark have not been observed so far. The neurone marked by 'U' was also in *r-PPG*, but could not be identified completely. See Fig. 1 for the names of the peripheral nerve bundles. See Fig. 5a for the connexion from the *r-PP 12* neurone, and also Fig. 6a, b for the pairs of *V 5* and *r-PP 1*, and *r-PP 6* and *r-PP 2*, having common input source. The signatures 'sim' and 'alt' mean the similar pattern (observed in the pair of *r-PP 1* and *r-PP 2*) and the alternating pattern (observed in the pair of *r-PP 1* and *r-PP 7* shown in Fig. 7b), respectively.

neurones. The neurones *r-PP 2* and *r-PP 7* had the similar and alternating patterns of activity. The *r-PP 1* neurone inhibited directly the *r-PP 5* neurone which received numerous IPSPs exclusively, as shown in Fig. 2b. An axon or axonal branches of the *r-PP 1* could not be found in any peripheral nerve bundles. The neurone *r-PP 1* may gather information from other neurones and from the periphery, and may distribute it to other ganglion cells, e.g. *r-PP 5*, but not to the periphery.

#### DISCUSSION

On the dorsal surface of the perioesophageal ganglia of *Onchidium verruculatum* there were found the somata of the giant neurones under the dissecting microscope. Each of them had always characteristic features, making it possible to identify the individual neurones, morphologically and electrophysiologically, and the interconnexions between and among them under direct visual control.

#### *Trans-synaptic connexions between the identified neurones*

In the present study four direct connexions were observed among the 190 pairs of neurones; i.e. in about 2% of tested pairs. The detectable direct connexions were

rarer than originally expected. Similarly, Willows & Hoyle (1968) mentioned that direct connexions were observed in 0.6% of cases in *Tritonia*. Why were they so rare?

The intracellular recordings were obtained from the soma of the neurone. But the locations of synapses are thought to be on an axon or axonal branches, as reported by Gerschenfeld (1963), Tauc & Hughes (1963), and Gorman & Mirolli (1969). Since the recording site in the soma might be remote from the synaptic area on the axon, PSPs in the subsynaptic area might not be fully recorded and observed from the soma. Therefore, their analysis is likely to be complicated, and few direct connexions were found in the records obtained from the soma.

If some synaptic contacts are on individual axonal branches, spike trains along the respective branches are affected separately by inputs from the particular synapses on them. Therefore, different information may be selectively conveyed through the separate branches of the single neurone, as supposed by Tauc & Hughes (1963). This speculation is very useful for the study of information-processing in the nervous system, because the speculation may offer the possibility that neural information is transformed by the microneural networks within single neurones.

#### *Activity pattern and neural network*

Since individual neurones have characteristic input-output pattern available for their identification, the pattern may correlate with the nature and functional role of the individual neurones. Every neurone is connected with other neurones and participates in composing neural networks. Therefore, the input-output pattern of the neurones must be discussed with regard to the structure of the network.

The alternating and periodic discharge patterns were demonstrated on a reciprocally inhibitory pair of neurones by Harmon (1964). Kandel *et al.* (1969) found the alternating activity pattern on a closed neural chain probably connected by mutually inhibitory connexions in *Aplysia*. If one neurone with an endogenous burst activity inhibited the other neurone and this could elicit rebound discharges during the silent intermissions of the former neurone, their firing pattern might be alternating. Furthermore, Kling & Székely (1968) and Suzuki, Katsuno & Matano (1971) demonstrated periodic discharge patterns of the simulated neurones ordered in a ring, with cyclic inhibition and mutual inhibition between neighbouring neurones.

The neurones *r-PP 1* and *r-PP 7* also showed the alternating discharge pattern (Fig. 7*b*). However, since they did not accept inhibitory synaptic inputs, they might be inhibited not by direct mutual inhibitory connexions but via a group of interneurones. It may be very difficult to identify the neural network including them, because detectable direct connexions were rare. The situation may be the same for the pair of neurones *r-PP 1* and *r-PP 2*, revealing the similar activity pattern.

#### *Pacemaker activity and trans-synaptic inputs*

Many of giant neurones, e.g. *V 1* and *r-C 1*, fired without apparent excitatory synaptic inputs, as shown in Figs. 2*a*, 3. Although the neurone *r-PP 5* in Fig. 2*b* accepted numerous IPSPs exclusively, it fired repetitively. Those neurones were thought to have an endogenous pacemaker activity, producing spike discharges automatically, as reported by Junge & Moore (1966), Frazier *et al.* (1967), Alving (1968), and Chalazonitis (1968). The neurone *r-C 1* showed a burst pattern of 15–17 spikes

occurring regularly with silent intermissions of about 5 sec, without synaptic inputs. Its burst activity was correlated with the slow fluctuation of membrane potential as observed in Fig. 3. When the membrane of the spontaneous bursting neurone was depolarized, interburst intervals became shorter and the burst firing sometimes changed to regular firing. When hyperpolarized, to the contrary, the interburst intervals became longer. These effects were also observed by Frazier *et al.* (1967) and Arvanitaki & Chalazonitis (1968) on the giant neurones of *Aplysia*. It was supposed there might be an endogenous burst activity affected by the change in the membrane potential. When burst activity was most pronounced, regular spike discharges of high rate could be elicited.

The interspike intervals of the neurone *r-PP 5* became longer with increase of arriving IPSPs. The intervals depended on the number of IPSPs. Observation of the input-output relation of the giant neurones suggested that output intervals might be determined by the number and the pattern of synaptic inputs (unpublished). The synaptic inputs may modulate the regular spike train arising from the intrinsic pacemaker activity, and a modulated spike train may be transferred to the other neurones as neural information to each of them. By this mechanism the neurones may transform neural information as discussed by Frazier *et al.* (1967). Speculation on the modulating effect of synaptic inputs on the endogenous pacemaker activity may be compatible with the finding of Alving (1968); i.e. the isolated soma of the pacemaker neurone of *Aplysia* could elicit action potentials, and the spike trigger zone and the pacemaker locus were on the soma of the neurone.

#### *Networks and the functional significance of them*

In *Onchidium* the neurone *l-PP 1* is possibly concerned with the movement of the mantle. A neural network composed of neurones is thought to be a subsystem of the nervous system and to play a part of the function of the whole nervous system. Recent investigations have disclosed that the particular neural networks in the central and peripheral nervous system controlled some stereotyped behaviour patterns of *Aplysia* (Kupfermann & Kandel, 1969; Kupfermann *et al.* 1971; Weevers, 1971), of *Spisula* (Prior, 1972 *a, b*), and of *Tritonia* (Willows & Hoyle, 1968). For the study of behaviour in relation to single-unit activity our identified neurones may be very useful material, because we can stimulate and record the activity of wanted neurones selectively. Moreover, from the same reason mentioned above, the identified neurones and networks may be used for the study of the plasticity of neuronal and synaptic function with regard to learning and neural memory.

#### SUMMARY

1. The marine pulmonate mollusc, *Onchidium verruculatum*, has many giant neurones in the perioesophageal ganglion complex.
2. Twenty of them were visually identified by their relative locations, sizes, and pigmentations under the dissecting microscope, and electrophysiologically by their input-output patterns.
3. The relationship among and between the giant neurones was investigated on 190 pairs of neurones, arbitrarily paired from 20 neurones, by intrasomatic recordings.

4. There were two excitatory and two inhibitory direct connexions. There were two pairs receiving synchronous excitatory inputs and two pairs receiving synchronous inhibitory inputs. Two pairs of neurones showed a similar activity pattern and one pair showed an alternating activity pattern.
5. A tentative neural network was constructed for one of identified neurones, *r-PP 1* in the *r-PPG* (right pleuroparietal ganglion).
6. The results and the firing patterns of neurones are discussed.

The author would like to express his deep gratitude to Professor R. Suzuki and Professor K. Murata for their encouragement and suggestions throughout the investigation.

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