IDENTIFICATION OF NEURONAL CONNECTIONS
IN THE CENTRAL NERVOUS SYSTEM
OF ONCHIDIUM VERRUCULATUM

Yoshifumi Katayama

Division of Bioelectronics, Institute for Medical and Dental Engineering,
Tokyo Medical and Dental University, Tokyo, Japan

It is important for the study of information processing in the nervous system to know neuronal connections as well as characteristics of neural signals conveyed by its elements. Neuronal connections in the abdominal ganglion of Aplysia have been studied by many authors1-8). Some of them1,4-8) recorded simultaneously spontaneous activity of two or more neurons with intracellular electrodes and identified several connections among them. The distribution of axonal branches was also studied by antidromic stimuli1,5,6,7).

Hughes3) stimulated a ganglion cell intracellularly and recorded extracellularly connective or commissure responses which had been triggered by spikes of the ganglion cell stimulated. By applying an averaging technique, he could demonstrate the existence of axonal branches in the connective or the commissure by the presence of a spike in an averaged response. Thus the averaging technique has been used successfully to identify output channels of the nerve cell, but so far has not been used for a study on input channels centering on an identified neuron.

In the present paper, the averaging technique was applied to a study on the neuronal connections in the central nervous system of Onchidium verruculatum. Some results indicating representative neuronal connections could be shown between ganglion cells and peripheral nerve trunks. Emphasis was placed on revealing input channels as well as output channels.

MATERIALS AND METHOD

1. Materials and their anatomy

The central nervous system of the marine pulmonate mollusc, Onchidium verruculatum, is around its oesophagus and called a perioesophageal ganglion complex. This complex was isolated carefully with its peripheral nerve trunks and immersed

Received for Publication June 13, 1970

711
in the filtrated sea water which was circulated quietly (2-5 ml/min). Its temperature was maintained between 15°-17°C.

The ganglion complex is composed of seven ganglia and five of them can be seen from dorsal side, as shown in Fig. 1. On the dorsal surface of the ganglion complex, there are some large nerve cells, giant neurons, which are suitable for an electrophysiological study because of the large size of their soma (100-300 μ in diameter) and their superficial location. About thirty of them could be distinguished in every specimen by visual investigations; location, size and pigment of them. Each of giant neurons was numbered as illustrated in Fig. 1. The neuron was denoted as l-PP 1, r-PP 3, V 5, r-C 2 et al.; “l” and “r” indicate left and right, respectively. PP, V and C are abbreviations of names of ganglia; PP (pleura-parietal), V (visceral) and C (cerebral) ganglion. The number consists with the number of each neuron in Fig. 1. Main peripheral nerve trunks are also shown in Fig. 1.

![Diagram of the perioesophageal ganglion complex of *Onchidium verruculatum.*](image)

**Fig. 1.** The dorsal view of the perioesophageal ganglion complex of *Onchidium verruculatum.* CG: cerebral ganglion; PPG: pleura-parietal ganglion; VG: visceral ganglion; PG: pedal ganglion which cannot be seen. PaN 1, 2 and 3: parietal nerve; IN: intestinal nerve; GN: genital nerve (left and right); AN: aorta nerve. “r” and “l” represent right and left, respectively. Identified neurons were numbered in this figure.

2. **Method**

Glass capillary microelectrodes for intracellular records were filled with 2 mol K-citrate and had a resistance of 20-50 megohms in sea water.

**Fig. 2** shows the schema of experiments. One microelectrode was inserted into the soma of a neuron under a binocular microscope and connected with a dc amplifier of high input impedance with a field effect transistor (FET). Amplified intracellular potential was displayed on a CRT (VC-7; NIHON KOHDEN), recorded on a magnetic tape (R-400; TEAC) and led to a digital computer (LINC-8; DEC) for data processing.

Single electrical stimuli were delivered to peripheral nerve trunks once every 5-10 sec through platinum-wire bipolar electrodes. The strength and the duration of stimulus were controlled independently and the former was kept as weak as possible in order to prevent stimulus artefacts.

3. **The use of the averaging technique**

If a ganglion cell (A in Fig. 2) has its axon or axonal branch in a given nerve trunk (a in Fig. 2), stimuli applied to the trunk would produce an antidromic invasion of a spike into the cell soma A, as shown in Fig. 2 (1).
FIG. 2. Diagram showing experiments and neuronal connections (axo-axonic). Electrical stimuli were delivered to nerve trunks (a and b) by bipolar electrodes. The transmembrane potential of neurons (A and B) were amplified by a dc amplifier and displayed on a CRT; (1) an antidromic spike may invade into the soma of the neuron A in response to stimuli applied to the trunk a; (2) PSP may be evoked in the neuron A through a bisynaptic pathway from the trunk b; and (3) PSP may be evoked in the neuron B through a monosynaptic pathway from the trunk b. Solid line: EPSP and broken line: IPSP, respectively. See text.

If a cell B receives presynaptic inputs from the stimulated trunk b through a monosynaptic pathway, a stimulus on the b would produce postsynaptic potentials (PSP's) in the neuron B after a definite delay necessary for the spike initiation in a presynaptic fiber, for the conduction along the nerve fiber and for a synaptic transmission; FIG. 2 (3) shows excitatory PSP's (EPSP's) and inhibitory PSP's (IPSP's) by a solid line and a broken line, respectively.

The situation is more complicated in the case that the neuron A receives excitatory or inhibitory effects from an interneuron through a disynaptic pathway. When the neuron B receives only excitatory synaptic inputs from the trunk b, it is supposed that this neuron fires in response to stimuli on the trunk b. This firing would in turn produce PSP's in the neuron A, as expected from such a schema of disynaptic excitation. The potential fluctuation of the neuron A would be an elicitation of EPSP or IPSP after a long delay from every stimulus, as shown in FIG. 2 (2).

However, since each single stimulus might occasionally fail to fire a presynaptic spike or an antidromic one, it could not elicit a fluctuation in the soma membrane potential either transsynaptically or antidromically; i.e. there are neither PSP's nor antidromic spikes in the soma. The potential fluctuation was sometimes so small that it might be hidden among spontaneous PSP's coming from other sources, spontaneous spikes and electric noises within experimental setups. It is well known, however, that the averaging technique can improve a signal-to-noise (S/N) ratio. Even the smallest change in membrane potential would become more apparent by this technique, repeating a stimulus-response test.
Averaging was carried out on-line or off-line with the digital computer, LINC-8. Necessary parameters were set initially for each averaging procedure; R: sampling time, D: the beginning of calculation, and NO: the number of averagings. They are shown in Fig. 3-9. Averaged responses were displayed on the LINC scope and photographed on 35 mm films.

The possibility and the limitation of this technique would be discussed later in discussion.

RESULTS

I. Representative Samples

Four types of neuronal connections were found between peripheral nerve fibers and ganglion cells in this study, as shown in Fig. 3-6. Each of figures displays a representative case.

1. Axon or Axonal Branch

The membrane potential of the soma of the neuron, l-PP 5, changed in response to stimuli applied to the peripheral nerve trunk, GNi, as shown in Fig. 3 (a); every stimulus was delivered at the beginning of each sweep. The change triggered by the stimulus was averaged 32 times. Fig. 3 (b) displays an averaged response, in which a large wave may result from spikes invaded into the soma after 40 msec delay from a stimulus onset. The delay of 40 msec was comparable with that recorded in the case of ganglion cells of Aplysia1,3,6.

The neuron, l-PP 5, may send its axon or axonal branch into the nerve trunk, GNi, as illustrated in Fig. 3 (c).

Fig. 3. (a) Sample records from the neuron, l-PP 5, in response to stimuli applied to the nerve trunk, GNi. Cal.: 20 mV and 200 msec at upper-right of this figure. (b) The averaged response. Time scale: 100 msec. R: sampling time; D: the beginning of calculation; NO: the number of averagings. (c) The tentative representation of the relation between the neuron, l-PP 5, and the trunk, GNi, drawn from (b). See text.
2. Monosynaptic Connection

1) Excitatory Connection

The membrane potential of the neuron, V5, changed in response to stimuli on the trunk, l-PaN 1-3, as shown in Fig. 4 (a) and was averaged 64 times. The manifest change in the membrane potential could be obtained as Fig. 4 (b), in which the curve, representing an averaged response, begins to rise at 60-80 msec after a stimulus onset. This observed delay from the stimulus onset to the beginning of rising of the curve would account for the time delay necessary for the spike initiation in a presynaptic fiber, the conduction along the presynaptic fiber and the synaptic transmission from a presynaptic terminal to a postsynaptic cell. Therefore the positive slow wave in Fig. 4 (b) may express averaged EPSP's. No spike discharges were observed during this averaging procedure, hence there was no interference by them.

A presynaptic fiber in the trunk, l-PaN 1-3, may discharge in response to stimuli on this and in turn EPSP’s are evoked in the postsynaptic cell, V5, through an excitatory synapse as illustrated in Fig. 4 (c).

2) Inhibitory Connection

The neuron, l-PP1, responded to stimuli delivered on the trunk, GNr, as shown in Fig. 5 (a). The uppermost photograph may represent an antidromic spike. Responses to peripheral stimuli were averaged 32 times with the exception of the response accompanied by spikes and Fig. 5 (b) was got.

In Fig. 5 (b), a small positive wave precedes a large negative wave. The former, the positive wave, begins at 40 msec after a stimulus. This value is comparable with the latency necessary for the invasion of an antidromic
spike into the soma, as mentioned above. Therefore the positive wave may be considered to have been produced by blocked antidromic spikes. This is suggested by the uppermost record in Fig. 5 (a). On the other hand, the nature of the large negative wave may be of the monosynaptic IPSP's because of its polarity and its time course. A latency of about 80 msec after stimulus also supports this interpretation.

The tentative connections between the neuron, l-PP 1, and the trunk, GNr, are schematically drawn, as shown in Fig. 5 (c); the neuron may send its axonal branch into the GNr and receive a monosynaptic inhibitory pathway from the trunk.

3. Multisynaptic Connection

As shown in Fig. 6 (a), the neuron, l-PP 2, fired only immediately after each stimulus applied to the nerve trunk, l-PaN 1-3, but failed to fire sometimes. EPSP's were evoked between 150-200 msec after every stimulus. Fig. 6 (b) shows two notable waves. The early spike-like large wave may be due to action potentials elicited by monosynaptic excitatory inputs after a delay of 60-80 msec from a stimulus onset. The next positive slow wave seems to have been produced by EPSP's evoked through a multisynaptic (at least disynaptic) pathway, because of its latency (about 180-200 msec) and its form. This interpretation may be supported by the lowermost record in Fig. 6 (a), which shows probably the action spike induced by an excitatory synaptic input through a disynaptic pathway. In summary this neuron may receive excitatory inputs through mono- and disynaptic pathways from the trunk, l-PaN 1-3, as shown in Fig. 6 (c).
Connections between ganglion cells and peripheral nerve trunks were studied by the averaging technique in 14 specimens. Some tentative neuronal connections terminating on certain identified neurons were drawn on the basis of four representative results. Differences among individual specimens seemed negligible so far studied. FIG. 7-9 show schematically some tentative neuronal networks obtained from observations made on three identified neurons, l-PP 1, V 1 and V 5 (see FIG. 1). They are as follows.

1) l-PP 1

The neuron, l-PP 1, was the largest neuron in the left pleuro-parietal ganglion and seldom fired. A tentative neuronal network in FIG. 7 (e) could be drawn from FIG. 7 (a-d).

In the case of stimuli on the trunks, l-PaN 1-2, IN and GNl, averaged responses could indicate antidromic spikes invaded into the soma of the neuron (Fig. 7 (a-c)). Stimuli on the trunk, GNr, evoked monosynaptic IPSP's and antidromic spikes which could not invade in the soma (Fig. 7 (d)). From Fig. 7 (a), a multisynaptic connection might be supposed between this neuron and the trunk, l-PaN 1-2, but some questions arose as to interpretations on FIG. 7 (a), as mentioned below in DISCUSSION.

2) V 1

The neuron, V 1, belonged to the visceral ganglion and was medium in its size. It fired spontaneously with a high frequency and a regular interval of 300-500 msec.

Stimuli on the trunk, l-PaN 1-2, could evoke monosynaptic EPSP's as shown in FIG. 8 (a) and those on the IN, GNl and GNr could evoke spike
Fig. 7. Proposed neuronal connections centering on the neuron, l-PP 1. (a-d): Averaged responses; time scale: 100 msec and stimuli were delivered at the trunks, (a) l-PaN 1–2, (b) IN, (c) GNl, and (d) GNr, respectively. (e): A neuronal network, drawn from (a-d). -: inhibitory synapse and +: excitatory synapse. This neuron may receive excitatory inputs from an unidentified interneuron indicated by a question mark and receive also inhibitory inputs from the neuron I, unidentified. Broken lines express ambiguous neuronal connections.
FIG. 8. Proposed neuronal connections centering on the neuron, V 1. (a–d): Averaged responses. Time scale: 100 msec. See the legend of FIG. 7 (a–d) for the trunks stimulated. (e): A neuronal network; the neuron, V 1, may receive excitatory inputs monosynaptically from either the peripheral trunks or neurons indicated by a question mark through their axon-collaterals. The output channels of this neuron were not identified, as indicated by a question mark.
FIG. 9. Proposed neuronal connections centering on the neuron, V 5. (a-d): Averaged responses. Time scale: 100 msec. See also the legend of FIG. 7 (a-d) for the nerve trunks stimulated. (e): A neuronal network centering on the V 5. See the legend of FIG. 8 (e) for the neurons, indicated by a question mark in this figure.
discharges monosynaptically, as shown in Fig. 8 (b-d). Thus it is supposed from these averaged responses that the neuron, V 1, may receive monosynaptic inputs from either presynaptic fibers in peripheral trunks or other neurons indicated by a question mark in Fig. 8 (e); that is, they may send axonal branches into trunks and also send axon-collaterals to this neuron.

3) V 5

The neuron, V 5, was large and fired irregularly. Fig. 9 (a-d) shows averaged responses and Fig. 9 (e) shows a neuronal network centering on the V 5.

From Fig. 9 (a), it is clear that stimuli on the trunk, l-PaN 1-2, evoke antidromic spikes. Stimuli on the GNl evoked also antidromic spikes and moreover monosynaptic EPSP's as shown in Fig. 9 (c). When the trunk, IN, was stimulated, spikes were elicited monosynaptically (Fig. 9 (b)). In the case of stimuli on the GNr, only monosynaptic EPSP's were produced (Fig. 9 (d)). These monosynaptic inputs may result from either periphery or other ganglion cells through axon-collaterals indicated by a question mark as illustrated in Fig. 9 (e).

DISCUSSION

Some neuronal connections were identified between ganglion cells and peripheral nerve trunks by an averaging technique. In favourable cases neuronal connections could be identified by a single stimulus-response test. As mentioned above, however, the single stimulus-response test could not always be available for the identification of neuronal connections. Therefore many stimulus-response tests should be examined and each response was averaged. It is well known that the averaging technique is useful for the improvement of a S/N ratio, as mentioned previously.

If the cell under observation should discharge spikes during averaging procedure, a result might be definitely affected by them, leading to an incorrect interpretation. The case of Fig. 7 (a) may be one of such examples. The neuron, l-PP 1, seldom fired but fired repetitively only in response to stimuli on the l-PaN 1-2. The averaged response (Fig. 7 (a)) contains two kinds of waves in their nature; i.e. three or four slow waves and many noise-like small spikes. The former might be produced by EPSP's or the aggregations of spike discharges of the neuron in response to incoming excitatory impingements. The latter might result from spontaneous spikes and be observed from a stimulus onset to a next one. Therefore the distortion by these spikes would not be so serious and the slow wave might represent excitatory inputs; EPSP's. There is one more possibility, however, that repetitive discharges might be evoked by the delayed effects of EPSP's or by the endogeneous pacemaker activity excited by stimulations. Therefore
it is difficult to draw any definite scheme of neuronal connections only on the basis of this result in Fig. 7 (a). But it may be possible to compose a tentative network illustrated in Fig. 7 (e); the neuron, l-PP 1, may receive monosynaptic and multisynaptic (through an interneuron indicated by a question mark) inputs from the trunk, l-PaN 1-2.

When spikes are set up in response to stimuli, a poststimulus time histogram (PST histogram) may be useful for studying neuronal networks. But if spikes result from the pacemaker activity excited by stimuli as mentioned above, the PST histogram might not be available for our purpose, too. Moreover the neuron cannot fire when the neuron receives only inhibitory inputs or when excitatory inputs, if any, are too infrequent. The PST histogram cannot be useful in this case. However, the averaging technique can be used for such a case, because it reveals slow potential changes including IPSP's.

If peripheral stimulations evoke IPSP's in an interneuron, there will be less chance for the interneuron to fire. In this case PSP's would not be produced in follower neurons. Therefore no information about neuronal connections was obtained, which is the limitation for the averaging technique, but the same is true for the PST histogram method.

Influence by spontaneous discharges must be excluded as much as possible. Three attempts were made for this purpose. Firstly, stimulus-response tests accompanied by spikes were excluded from averaging as mentioned previously. Secondly, spikes in the soma of a neuron were suppressed by the transmembrane electric current which could hyperpolarize membrane potential. The effective strength of the current, however, had the possibility that the polarity of IPSP changed to the contrary. Thirdly, the number of averagings was increased. It is clear that the larger the number is, the lesser would become the influence of spikes which intervened during averaging procedure. But it was difficult to forecast how many times we should average to obtain distinct results. Actually, observing averaged responses, averaging was executed 16-64 times.

It is supposed that there may be axo-axonic connections in the case of giant neurons of Onchidium. In the spontaneous activity of the neuron, V 1, any synaptic input has not been observed so far. This may be interpreted as follows. Since synaptic connections are on its axon in a neuropile distant from its soma, EPSP's and IPSP's are too small to be observed in the membrane potential recorded at the soma. In the present paper, however, it was assumed that the neuron, V 1, might receive monosynaptic inputs as shown in Fig. 8. Thus by applying the averaging technique, very small changes in the membrane potential of the soma could be distinct in spite of the long distance between a synapse and the soma.

Fig. 7 shows that the neuron, l-PP 1, has axonal branches in peripheral
trunks and receives multisynaptic input pathways probably from the l-PaN 1-2 and a monosynaptic inhibitory input channel which comes from the trunk, GNr, associated with contralateral PPG and PG. But the origin and the nature of this inhibitory channel are not yet defined. There may be two possible interpretations: the one is that an inhibitory afferent may come from the trunk, GNr, and the other is that inhibitory effects may be exerted by an axon-collateral from an unidentified neuron marked as "I".

The neuron, V 5, receives several monosynaptic input pathways and sends output channels into several peripheral nerves, as illustrated in Fig. 9.

On the other hand, the neuron, V 1, receives inputs but does not send outputs to periphery, as shown in Fig. 8. This neuron may collect neural informations from other parts of nervous system and send them to neurons within a ganglion system and act as a distributor of neural informations.

For studying neural information processing, it may be required to investigate a nervous system from the viewpoint of system engineering; to determine the neuronal networks as mentioned in this paper and also the characteristics of the signals conveyed by neural elements. The averaging technique used in these investigations may be useful for the identification of neuronal networks.

SUMMARY

1. Investigations were made on the nervous system of Onchidium verruculatum, pulmonate mollusc. A microelectrode was inserted into the soma of the giant neuron on the dorsal surface of the perioesophageal ganglion complex in order to record the change in membrane potential.
2. Peripheral nerve trunks were stimulated electrically through bipolar electrodes. Each peripheral stimulus evoked the change in the membrane potential which were averaged by the digital computer, LINC-8.
3. The analysis of the averaged responses could disclose the modes of synaptic connections, including a direct connection; i.e. an axon or an axonal branch.
4. On the basis of the averaged responses, neuronal connections were deduced between peripheral nerve trunks and ganglion cells which could be always identified under a binocular microscope.
5. The possibility and the limitation of the averaging technique are discussed.

The author would like to express his deep gratitude to Professor Ryoji SUZUKI and Professor Kei-ichi MURATA for their encouragements and suggestions throughout investigations and to Professor Yasuji KATSUKI for his valuable advices and criticisms in preparing the manuscript. The author also would like to express his appreciation to Mr. Sadao MINAMI for the computer programme used.
REFERENCES


