Local interneurons and motoneurons involved in the control of abdominal postural movement in crayfish

by Tetsuo Toga

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CONTENTS

General Introduction

The problem of how animal movement is controlled by the nervous system has been of great interest in biology. Invertebrate animals provide a suitable model for studying the neuronal basis of animal behavior because of the structural simplicity of their nervous system.

In several invertebrate motor control systems, stimulation of single nerve cells has been shown to be a useful technique for analyzing animal behavior. Wiersma and Ikeda (1964) first reported that the rhythmic movements of swimmerets could be initiated by stimulation of nerve bundles isolated by fine dissection from the ganglionic connective in crayfish. A dissected bundle contained one or a few interneurons located in the connective. These interneurons could evoke well-coordinated motor patterns when stimulated. The interneurons that initiate coordinated movements were considered to be "commanding" movement (Wiersma and Ikeda 1964). Interneurons capable of evoking specific behavior were soon found in other invertebrates and were generally described as "command interneurons." This discovery led to the belief that the neuronal basis of animal behavior could be understood in terms of a model of command fibers.

Movements of abdominal segments of crayfish were also evoked by stimulation of other kinds of command fibers in the connective (Kennedy et al. 1966b). The abdominal positioning system has been investigated using extracellular techniques (Kennedy et al. 1966b,1967; Evoy and Kennedy 1967; Larimer and Kennedy 1969). Stimulation of different command fibers could evoke various positioning movements of the abdomen (Bowermann and Larimer 1974). Thus the positioning movements of the crayfish abdomen (flexion and extension) have been the most intensively studied behavior in invertebrates in terms of neuronal organization and interneuronal connection (Page 1982; Larimer 1988). It has been thought that activation of a single command fiber is sufficient to evoke a certain abdominal positioning movement.

Recently, this system has been investigated using intracellular techniques including dye-filling (see review, Larimer 1988). Several recent studies have indicated that the command fibers which control abdominal positioning behavior are organized into parallel and serial functional groups through their synaptic connections (Jellies and Larimer 1985; Larimer 1988; Murphy et al. 1989). These studies suggest that abdominal positioning interneurons are not "command fibers", as defined by Kupfermann and Weiss (1978). Murphy et al. (1989) hypothesized that abdominal positioning behavior was under the control of a large network of neurons each of which made a small contribution to the overall motor output. Although these previous studies have emphasized the significance of the interganglionic interneurons for postural control, the contribution of other kinds of neurons, for example intraganglionic interneurons, in the neuronal network hypothesized by Murphy et al. (1989) is unknown.

This thesis focuses on the neuronal network controlling abdominal positioning movements in crayfish to investigate the neuronal basis of animal behavior. I surveyed and identified local (intraganglionic) nonspiking interneurons which participated in the control of abdominal positioning behavior. This is the first identification of local interneurons in the abdominal posture system of this function. In chapter I, I will report the morphological and physiological characteristics of the newly identified interneurons, and discuss their possible role in the control of abdominal positioning behavior.

In chapter II, I tested the effect of whole or partial inactivation of neurons on their function by using a laser light epiillumination apparatus newly devised in our laboratory. Partial inactivation by spot illumination could ablate a particular region of a neuron. The technique of laser photoinactivation was adopted to investigate the characteristics of the abdominal postural motoneurons in crayfish. I will discuss the validity of the current method of laser light epiillumination from biological perspective.

In chapter III, I will report the significance of the postural motoneurons in the control of abdominal positioning behavior in crayfish. Abdominal postural motoneurons had premotor effects on their synergists in the ganglion. Physiological and morphological evidence for coupling among motoneurons was found. The functional significance of coupling will be discussed.

CHAPTER I

Local nonspiking interneurons involved in abdominal postural movement.

SUMMARY

Local interneurons of a bilateral structure were found in the fourth abdominal ganglion. The interneurons did not generate action potentials spontaneously at their resting potential or in response to current injection of either polarity or electrical stimulation of sensory pathways. Artificial depolarization of the interneurons by current injection caused an inhibition of the spontaneous spike discharge of abdominal postural motoneurons without spike generation in the interneurons themselves. Membrane potential change of the interneurons during fictive abdominal posture movement was measured to determine the actual possibility of involvement of the interneurons.

Depending on the distribution of neurites of the interneurons, two major morphological types could be distinguished. However, no significant difference between these two morphological types was found with respect to physiological characteristics.

In addition to these bilateral local nonspiking interneurons, I found unilateral local nonspiking interneurons which could evoke change in the activity of the abdominal postural motoneurons in the fourth abdominal ganglion. Consequently, these kinds of local nonspiking interneurons are considered to be part of the neural circuit controlling abdominal posture movement.

INTRODUCTION

The presence of intraganglionic (local) interneurons in the central nervous system was first reported by Retzius (1890) using the methylene blue staining method. Their structure was restricted to the ganglion, and there was no neuronal branch to other ganglia. The physiological function of the local interneurons, however, has been unknown for a long time. It was first reported in insects that a local interneuron could modulate the spike activity of motoneurons without its own spike generation (Pearson and Fourtner 1975). In crayfish, Heitler and Pearson (1980) reported the presence of local nonspiking interneurons which modulated the spike activity of swimmeret motoneurons in the fourth and fifth abdominal ganglia. Takahata et al. (1981a) reported that spike activity of uropod motoneurons in the sixth abdominal ganglion was under strong control of local nonspiking interneurons. Further studies (Paul and Mulloney 1985a,b; Reichert et al. 1982; Nagayama et al. 1984; Nagayama and Hisada 1987) have revealed the structure and function of local nonspiking interneurons. These previous studies, however, have been concentrated on the swimmeret and uropod motor control systems.

The abdominal posture control system has been one of the most intensively studied motor system in crayfish since Kennedy and Takeda's (1965) study. Postural movement of the abdomen results from the tonic contractions of the slow extensor or flexor muscles (Kennedy and Takeda 1965; Kennedy et al. 1966a; Fields 1966). It has been well established that the activity of the motoneurons innervating these postural muscles is effectively coordinated by interganglionic interneurons in the abdominal nerve cord (Kennedy et al. 1966b). The interneurons that initiate abdominal extension and flexion movement have been called "command fibers" after the terminology introduced by Wiersma and Ikeda (1964) for swimmeret movements in crayfish (see also Kennedy et al. 1966a; Evoy and Kennedy 1967). Most of the studies concerning these kind of interneurons have been performed using extracellular electrode techniques.

In the last decade, there have been some reports on abdominal postural interneurons in which the intracellular microelectrode technique was used (see review, Larimer 1988). These interneurons are mostly interganglionic, except for two (each encountered only once) which are local (Jellies and Larimer 1985). The two local interneurons were found to be nonspiking, as reported in a various motor control systems of arthropods (see Siegler 1985). In the abdominal posture system, however, it is still unknown to what extent local nonspiking interneurons participate in the control of motoneuron activity.

I surveyed the local nonspiking interneurons involved in the control of abdominal posture movement of crayfish. I repeatedly encountered and identified a set of local nonspiking interneurons which could modulate the spike activity of the postural motoneurons in the same fashion in different preparations.

In this chapter, I will present the morphological and physiological characteristics and the possible role of local nonspiking interneurons in the abdominal posture control system.

MATERIALS AND METHODS

1. Animals

All experiments were carried out on adult crayfish *Procambarus clarkii* Girard of both sexes. There were no obvious differences in the obtained results between male and female animals. Crayfish which were used for experiments had a body length of 8-11cm from rostrum to telson. They were obtained commercially (Sankyo Lab Service Co., Ltd., Tokyo, Japan), and kept in the laboratory tanks before use.

2. Preparation

Abdominal nerve cord preparations were used for experiments. The abdomen was cut apart from the thorax. All swimmerets were removed. Sternal ribs and the cuticle overlaying the nerve cord were removed. The nerve cord was excised and pinned dorsal side up on a silicon rubber-lined dish filled with crayfish saline (Harreveld 1936) at room temperature. The ganglionic sheath was left intact.

Whole animal preparation was also used for recording of neuronal activity during fictive abdominal posture movement of crayfish (Fig.1; see also Takahata and Hisada 1986a). The animal was suspended in the air by a steel rod connected to its carapace. No leg substratum was provided except in the case where the stimulus was applied for reflexively eliciting the abdominal extension movement (see Results). The abdomen was fixed on a cork board at a horizontally extended posture. The dorsal cuticles on the third, fourth and fifth segments were removed. The intestine was severed just anterior to the constriction using thread. Dorsal musculature of slow and fast extensor muscle and fast flexor muscle was removed to expose the fourth abdominal ganglion. The ganglion was stabilized on a stainless plate. The abdominal cavity was frequently flushed with crayfish saline.

3. Extra- and intracellular recording and staining

Spike activity of postural motoneurons was monitored extracellularly with suction electrodes. These electrodes were placed on the second roots or on the superficial third roots in the fourth abdominal ganglion. The second root contains the axons of tonic abdominal extensor motoneurons (Fields et al. 1966; Kennedy et al. 1966a). The third root contains the axons of tonic abdominal flexor motoneurons (Kennedy and Takeda 1965). These electrodes could also be used for electrical stimulation of the nerves by changing the switches which were interposed between these electrodes and preamplifiers. Nerve roots were stimulated with rectangular current pulses from an electronic stimulator via an isolator.

A glass capillary microelectrode for intracellular recording was driven across the sheath of the ganglion. The electrode contained the dye solution of 3% Lucifer yellow in 0.1 M $LiCl_2$ or 5% in 1 M $LiCl_2$ (Stewart 1978). Tips of the electrodes were immersed in India ink to facilitate identification of the electrode tip under the dissecting microscope.

The criteria by which a penetrated neuron was classified into a nonspiking neuron were as follows: 1) no injury spikes were observed when the neuron was penetrated; 2) no spikes were observed either spontaneously or in response to sensory afferent stimulation; 3) activity of tonic motoneurons was changed by current injection into the penetrated neuron without its own spike generation; 4) no rebound spikes were observed after injection of hyperpolarizing current less than 10nA into the neuron. When the electrode seemed to be in a tonic motoneuron, it was identified by antidromic stimulation and by 1:1 correspondence between the intracellularly and the extracellularly recorded spikes.

RESULTS

We stained 44 local interneurons in the fourth abdominal ganglion of 42 different preparations. These local interneurons did not generate their own spikes. Among these neurons, 42 neurons had their neurites in bilateral hemiganglia, and 2 neurons showed unilateral neurite distribution.

Bilateral local nonspiking interneurons

1. Morphological characteristics

The stained neurons show bilateral distribution of their dendritic branches extended on both sides in a ganglion. These bilateral branches were connected by a thick dorsal process which runs transversely. No axonal structure was observed. The cell body was always located in the ventrolateral portion of the ganglion and was connected to the neuropilar processes via a thin neurite. These local bilateral interneurons are called LB cells in this thesis.

The LB neurons could be further classified into two morphological types. LB1 cells (n=29) extended their branches on both the ipsilateral and contralateral side of the ganglion to their somata, with a few dendrites near the midline (Fig.2A1). The dorsal process had a diameter of 9.3±1.2 μm (mean±S.D. n=12 out of 29 neurons). When the shape of the soma was considered to be an ellipse, the minor axis of the soma was 23.2±9.5 μm in length and the major axis was 31.6±9.5 μm in length (mean±S.D. n=11 out of 29 neurons). On two occasions, two LB1 cells whose somata were in the same hemiganglion were stained in the same preparation (Fig.2A2). LB1 cells showed morphological variability from animal to animal (Fig. 2C).

There was another type of morphology of LB cells. This type of neuron is called LB2 cell (n=13). These neurons also had two main branching regions. The location of these branching regions was different from that of LB1 cells. LB2 cells had an asymmetrical distribution of dendrites, mainly on the contralateral side to

the soma and near the midline (Fig.3A). These two branching regions were connected by a thick dorsal transverse process of which the diameter was 9.2±1.9 μm (mean±S.D. n=5 out of 13 neurons). The soma of this type of the neuron was also located in the ventrolateral portion of either side of the ganglion. The soma was usually elliptical in shape with a minor axis of 24.6±8.4 μm and a major axis of 29.7±7.6 μm (mean±S.D. n=4 out of 13 neurons). This type of cell showed little variability among animals.

2. Physiological characteristics

Current injection into LB cells modulated spontaneous discharge rates of the motoneurons in the second roots and the superficial third roots of the fourth abdominal ganglion. Morphological differences between LB1 and LB2 cells had no relation to their physiological characteristics.

Injection of depolarizing current into LB cells inhibited spontaneous spike discharge of a certain tonic unit in the second roots, whereas hyperpolarizing current injection had no discernible effect on the unit (Fig. 4). The spontaneously firing unit had the largest extracellular spike amplitude in the root. The unit was easily distinguished from other units by its spike amplitude, and was identified as slow extensor inhibitory motoneuron (identification of motoneurons is presented below). Inhibitory effect of LB cells on the motoneuron was graded manner and depended on the intensity of injected current (Fig. 4B).

The superficial third root contains axons of the slow flexor motoneurons (Kennedy and Takeda 1965). Spontaneous firing of spikes recorded from the roots was inhibited when a depolarizing current was injected into LB cells (Figs 2B2, 3B2). Hyperpolarizing current injection usually had no noticeable effect, except in a very few cases where weak excitation was observed. These inhibited units in the roots seemed to be slow flexor excitatory motoneurons (see below). The slow flexor inhibitory motoneuron was not excited by LB cells. Neither type of LB cell had any visible effect on the fast motoneurons in either the second or the third

roots, on the swimmeret motoneurons in the first roots, or on the interganglionic interneurons in both the 3-4 and 4-5 connectives.

Resting potential of LB cells was 61±11.8 mV (mean±S.D. n=11 out of 42 neurons). In the resting state, hyperpolarizing postsynaptic potentials (PSPs) were spontaneously superimposed on the resting potential level. Amplitude of the PSPs increased by depolarization of LB cell by current injection, while hyperpolarization decreased the amplitude and reversed the polarity of the PSPs. The PSPs, therefore, were chemically mediated inhibitory postsynaptic potentials.

Electrical stimuli were applied to the first or second roots to mimic sensory input to LB cells from the periphery, since these roots contain afferents from the peripheral sensory organs (Hughes and Wiersma 1960; Wine and Hagiwara 1977). Stimulation of either of the roots evoked excitatory postsynaptic potentials on LB cells, but could not elicit spikes in the cell (Fig.5A). Electrical stimulation was also applied to interganglionic interneurons running through the ganglionic connective. The hemiconnective between the third and fourth ganglia was divided into three or four bundles with a fine needle. Stimulation of each bundle could inhibit the spike discharge of the slow extensor inhibitory motoneuron (abbreviated as EI hereafter), and evoke depolarization of LB cell (Fig.5B). The effect of bundle stimulation was not canceled by the injection of hyperpolarizing current in LB cells (Fig.5B). The reciprocal effects of the stimulation of EI firing and membrane potential change in LB cells were consistent with the mode of operation of LB cells for abdominal posture movement.

3. Activity of LB cell during fictive abdominal posture movement

The membrane potential change in LB cells during the 'fictive' abdominal posture movement was studied using the whole animal preparation (Fig.1; see also Takahata and Hisada 1986a). LB cells were penetrated in three different animals. Fictive abdominal posture movement was induced by forcibly moving the walking legs that were left free in the air (Larimer and Eggleston 1971; Page 1981). During

the fictive abdominal extension, as judged by an increase in activity in the extensor excitatory motoneurons and a decrease in activity in the flexor excitatory motoneurons, the LB cell showed sustained depolarization as long as the extension pattern of motor activity continued (Fig.6).

The maximal amplitude of this depolarization was about 20mV, which was sufficient to suppress the postural motoneurons in the current injection experiment (Figs 2B, 3B). However, we could not determine whether the depolarization was the exclusive cause of suppression of the flexor excitor activity.

Identification of motoneurons affected by LB cells

The unit with the largest extracellular spike amplitude among the tonically discharging units in the second root was identified by its physiology and morphology. This was the only unit that was inhibited by artificial depolarization of LB cells by current injection (see above). The frequency of spontaneous discharge of this neuron ranged from 2 to 9 imp./s.

I stained the motoneuron intracellularly in 14 different animals. This motoneuron was identified as the slow inhibitory motoneuron innervating the superficial extensor muscle (Kennedy et al. 1966a; Fields et al. 1967) on the basis of its structural characteristics (Fig. 7A).

The motoneuron had a bilateral structure of its dendrites and contralateral soma to the root of exit. When the shape of the soma was considered to be an ellipse the minor axis was 47.7±7.0 μm in length and the major axis was 61.3±10.4 μmin length (mean±S.D. n=7 out of 14 neurons). These morphological features, the bilateral structure and soma size, correspond with those of the slow extensor inhibitory motoneuron which was previously reported (Wine and Hagiwara 1977; Miall and Larimer 1982). The largest unit in the second roots, therefore, was identified as the inhibitory motoneuron.

The superficial third root contains only six axons of slow flexor motoneurons (Sokolove and Tatton 1975). The motoneurons have been numbered

sequentially according to increasing spike heights as recorded in the motor root (Kennedy and Takeda, 1965; Wine et al., 1974). In the extracellular recording from the root, some units showed spontaneous spike firing of different amplitudes. We identified these spontaneously active units as the slow flexor excitatory motoneurons, because the slow flexor inhibitory motoneuron had been reported to usually be silent and to operate in reciprocity with the excitatory ones (Kennedy and Takeda 1965; Kennedy et al. 1966a).

Unilateral local nonspiking neurons

Two unilateral local nonspiking interneurons were found in the fourth abdominal ganglion. Both neurons caused a change in the spike activity in the second roots by current injection into the cell, but their physiologies were different.

One example was dye-coupled interneurons (Fig. 8). Injection of depolarizing current into the interneurons decreased the spontaneous discharge rate of EIs and slow flexor excitatory motoneurons in each root, but injection of hyperpolarizing current had no discernible effects. Electrical stimulation of sensory afferents in the second roots elicited no response in the interneurons.

The other example was also a pair of dye-coupled interneurons. Injection of a small amount of depolarizing current into the cells (+3nA≤) had no effect on the activity of the second root while injection of a small amount of hyperpolarizing current (-1nA≤) activated the discharge of some units but not the EI in the second root (Fig. 9). Electrical stimulation of the second root caused hyperpolarization on the membrane potential level of the interneuron.

In addition to these examples, there was a unilateral local nonspiking interneuron which modulated the spike activity of the units in the second roots in the fifth abdominal ganglion (Fig. 10). This interneuron had bi-directional functions as to the control of the spike activity of the second roots motoneurons. Injection of depolarizing current inhibited the spike discharge of units in the

second roots while a hyperpolarizing current injection activated the units. Current injection of either polarity, however, had no effect on the activity of the third root.

DISCUSSION

In this study, I surveyed local interneurons in the fourth abdominal ganglion, and found local nonspiking interneurons which altered the spike activity of motoneurons innervating abdominal postural muscle (Kennedy and Takeda 1965; Kennedy et al. 1966a). In the following sections, I will discuss the possible role of these newly identified local nonspiking interneurons in abdominal posture movements. Subsequently, I will attempt to compare the local nonspiking interneurons with other previously reported local nonspiking interneurons in the abdominal ganglionic chain in crayfish.

1. The possible role of the local nonspiking interneurons

Artificial depolarization of the newly identified local bilateral nonspiking interneuron (LB cell) by current injection inhibited the spontaneous spike discharge of the slow extensor inhibitory motoneuron (EI) (Kennedy et al. 1966a; Fields et al. 1967) and the slow flexor excitatory motoneurons (Kennedy and Takeda 1965; Kennedy et al. 1966a). The inhibitory effect of LB cells on these postural motoneurons suggests the possible role of LB cells in the abdominal posture control system.

Postural movement of crayfish abdomen results from the tonic contraction of the slow extensor or flexor muscles, and relaxation of the antagonistic muscle (Kennedy and Takeda 1965; Fields et al. 1966; Kennedy et al. 1966a). It is clear that both flexion and extension movements are reciprocally evoked both within and between muscle groups; that is, excitation of the excitatory motoneurons and suppression of the inhibitory motoneuron occur in the activated muscle, and activation of the inhibitor and inhibition of all excitors in the inhibited muscle (Kennedy et al. 1966a). During the fictive abdominal movement, reciprocal activation occurred between antagonistic groups of motoneurons (Fig. 6). Suppression of the activity of EIs would be necessary for the activation of the slow extensor muscle, and suppression of slow flexor excitatory motoneurons

would impede the activation of the slow flexor muscle which would operate antagonistically to extension movement. The simultaneous inhibition of two sets of motoneurons may assure the extension movement of the abdomen.

Electrical stimulation of a divided connective bundle inhibited the discharge of EIs and evoked depolarizing PSPs on the LB cell (Fig. 7). The evocation of depolarizing PSPs on LB cells indicates that LB cells receive depolarizing synaptic input conveyed by interganglionic interneurons from other ganglia or the brain. The mode of activation of LB cells and inhibition of EIs by stimulation of connective bundles were consistent with the mode of operation of LB cells in the experiment of current injection. Electrical stimulation of the first and second roots which contain afferent neurons from peripheral sensory organs (Hughes and Wiersma 1960; Wine and Hagiwara 1977) also evoked EPSPs on the LB cell. This observation suggests that either central or peripheral input can activate LB cells for their operation. Sustained membrane depolarization of LB cells during fictive abdominal movement indicates that LB cells participate in a neuronal network controlling abdominal postural movement. Activation of LB cells by stimulation of interganglionic interneurons, and the bilaterally coordinated output of LB cells, which is also well coordinated in terms of the antagonism between flexor and extensor activities, seem to be consistent with the idea that local interneurons act as driver interneurons interposed between the interganglionic command neurons and the motoneurons (Evoy 1967; Evoy and Kennedy 1967). However, the observation in the study, that local nonspiking interneurons operate not by exciting the extension circuit but by inhibiting the flexion circuit, suggests that the interneurons act as part of an auxiliary or modulatory circuit which facilitates the extension movement by inhibiting the neural activity operating antagonistically to that movement. The neural circuit which actively drives the extensor motoneurons during the extension movement remains to be investigated.

I also obtained two examples of unilateral local nonspiking interneurons. One inhibited the spontaneous discharge of EI and slow flexor excitatory motoneurons by its artificial depolarization. The other interneuron activated some unidentified units in the second root by its hyperpolarization. These interneurons can also facilitate or produce abdominal extension movement by their membrane polarization. Both unilateral and bilateral local nonspiking interneurons seem to be involved in the facilitatory control system of abdominal extension movement (Fig. 11).

2. Comparison with other local nonspiking interneurons

Two examples of local interneurons (LIs) have been reported previously in the third and fifth abdominal ganglia (Jellies and Larimer 1985), with bilateral morphology similar to that of LB1 cells and quite different from that of LB2 cells. Although this suggests the possibility that the LB1 cells are serially homologous with the LI cells, the difference in motor effects between them makes this possibility less likely: depolarization of the LB1 cells decreased the spike activities of both the slow extensor inhibitor and slow flexor excitors, whereas depolarization of the LI cells caused an increase in spike firing of the flexor inhibitory motoneuron and a decrease in the flexor excitatory motoneurons. Since no increase in the activity of the flexor inhibitor was observed in any of our preparations, we concluded that the LB1 cells were not identical to the LI cells. The present study thus shows that there are several types of local nonspiking interneurons, at least 4 types, involved in neuronal control of abdominal posture. The results of the previous report by Jellies and Larimer (1985) and this study are consistent in that the bilateral nonspiking interneurons do not excite the abdominal extension system but inhibit the flexion system. This also supports my hypothesis that the local bilateral nonspiking interneurons in the abdominal posture system are not directly involved in the mediation of extension movement.

Many unilateral local nonspiking interneurons in the sixth abdominal ganglion can modulate the activity of uropod motoneurons by injection of current of either polarity, and some of them can operate by injection of current of both polarities (Takahata et al. 1981a; Nagayama et al. 1984; Nagayama and Hisada 1987). The bi-directional operation of the unilateral nonspiking interneurons may be due to their continuous release of transmitters at their resting potential (Nagayama et al. 1984; Hisada et al. 1984; Nagayama and Hisada 1987; Hisada 1989). Hyperpolarizing current injection into LB cells had no effect on the spike activity of the abdominal postural motoneurons. LB cells may be inactive at their resting state, and may release their transmitters only at their depolarized state. The mode of transmitter release of LB cells is similar to that of the premotor local nonspiking interneurons of a bilateral structure in the sixth ganglion. The bilateral interneurons could exert premotor effect when they were injected with depolarizing current, and seemed to be inactive at their resting potential (Nagayama and Hisada 1988). The bilateral local nonspiking interneurons may have the common feature of transmitter release.

Unilateral local nonspiking interneurons of similar structure are closely packed together to form a cluster in the sixth ganglion (Hisada et al. 1984). There were two preparations in which two LB cells of similar structure were closely packed in the fourth ganglion (Fig.2C). LB cells may also form a cluster in the ganglion. The exact number of the cells in the ganglion, however, is an open question.

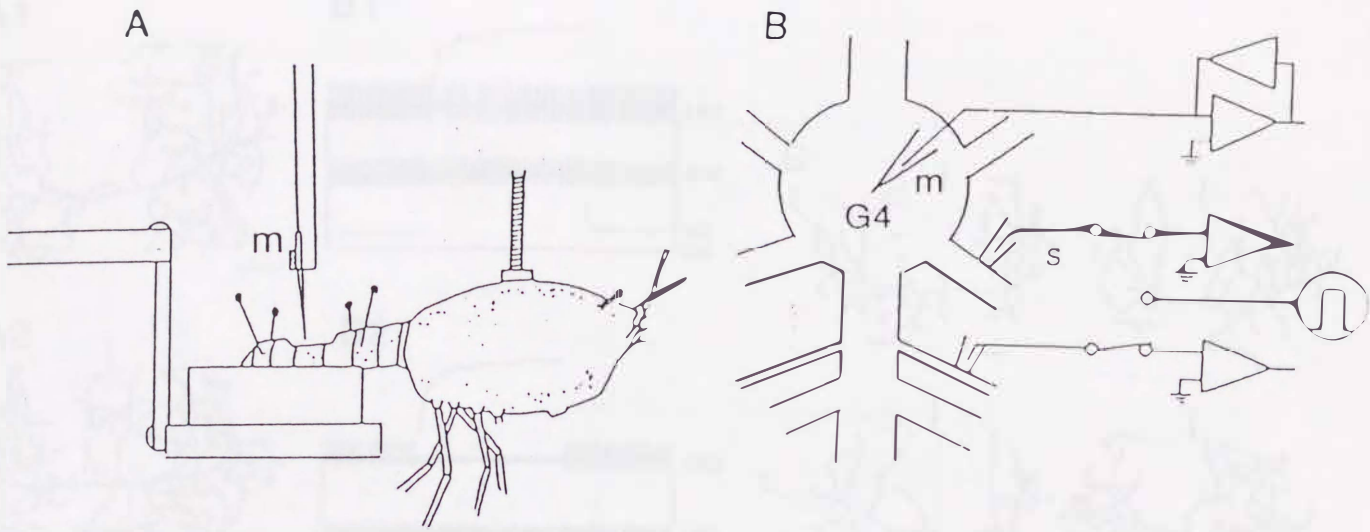

Figure 1. Experimental setup in the study. A: dorsal preparation for intracellular recording of LB cell activity during the fictive abdominal movement. The animal was hung in the air by a metal rod on its cephalothorax. The abdomen was pinned onto a cork board. No leg substratum was provided except when the stimulus was applied for reflexively eliciting the abdominal extension movement. The microelectrode (m) is shown but other electrodes for extracellular recording are not shown. B: typical arrangement of electrodes in isolated nerve cord preparation. Suction electrodes (S) were place on nerve roots.

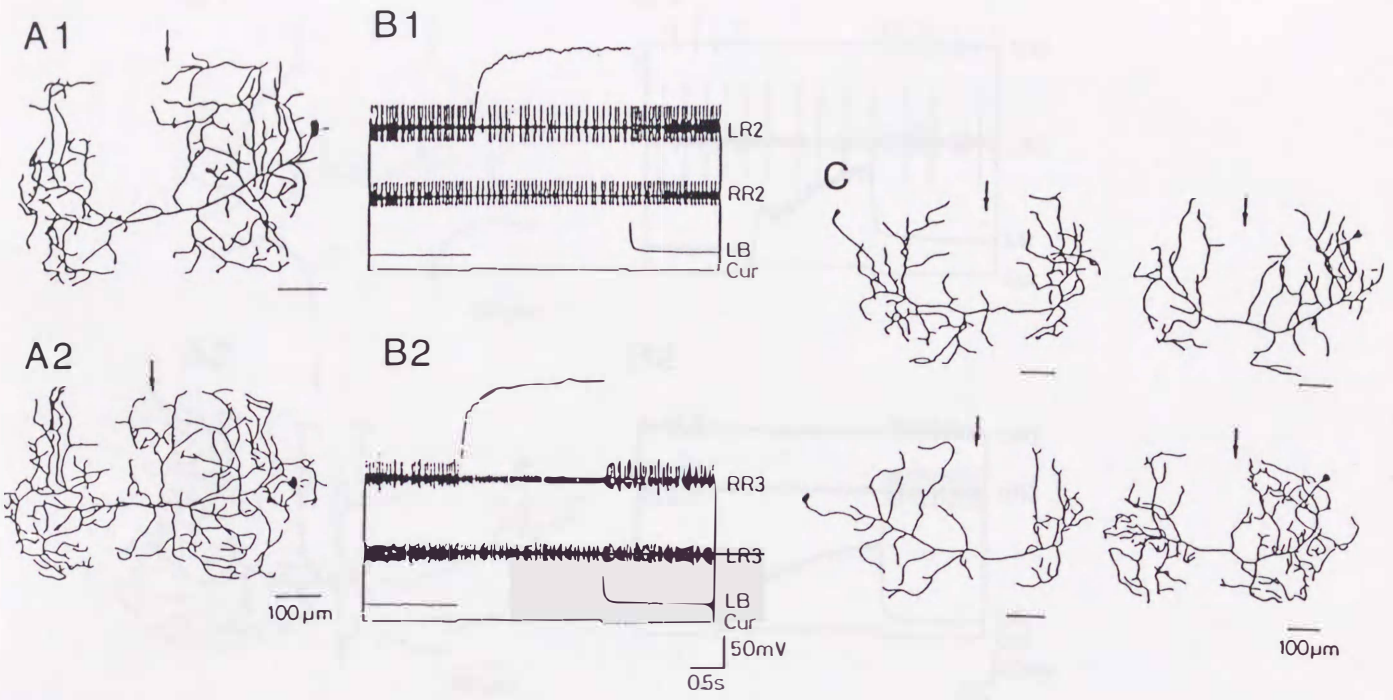

Figure 2. Structure of LB1 cells and the effect of current injection on spike activity of the abdominal postural motoneurons. (A1) Structure of LB1 cell stained with Lucifer yellow. (A2) Two LB1 cells stained simultaneously. Anterior is at the top. Arrows indicate the midline. (B1,B2) Effect of current injection into the cell shown in A1. (B1) Intracellular injection of 3nA depolarizing current inhibited spontaneous spike discharges of the second roots on both sides. (B2) 3nA depolarizing current also decreased the spike discharge rate of the third roots. LR2, motor activity in the left second root; RR2, the right second root; LB, intracellular recording from the LB cell; Cur, monitor of intracellular current injection. (C) Variability of morphology among LB1 cells believed to be homologues.

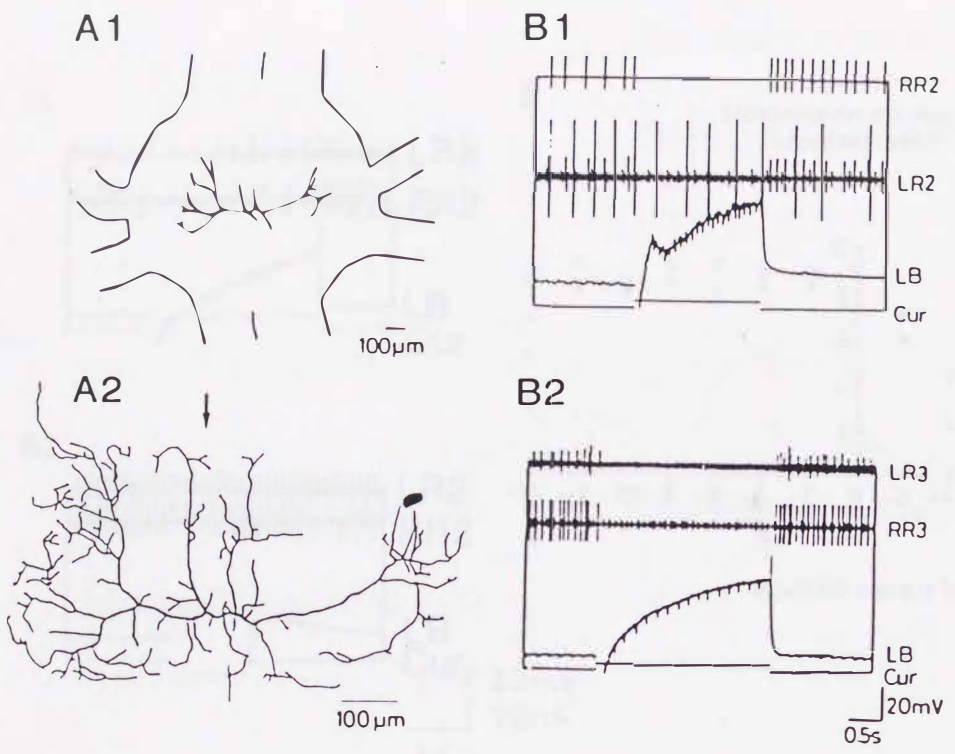

Figure 3. Structure of LB2 cell and the effect of current injection. (A1) Location of LB2 cell stained with Lucifer yellow in the fourth abdominal ganglion. (A2) Reconstructed structure of the cell shown in A1. Anterior is at the top. The arrow indicates the midline. (B1) Intracellular injection of 5nA depolarizing current into the cell shown in A inhibited spontaneous spike discharge of the slow extensor inhibitors in the second roots. Second largest unit in the LR2 (second trace) is the slow extensor inhibitor. (B2) 4nA depolarizing current injection in the other LB2 cell inhibited spike discharge of the third superficial roots on both sides.

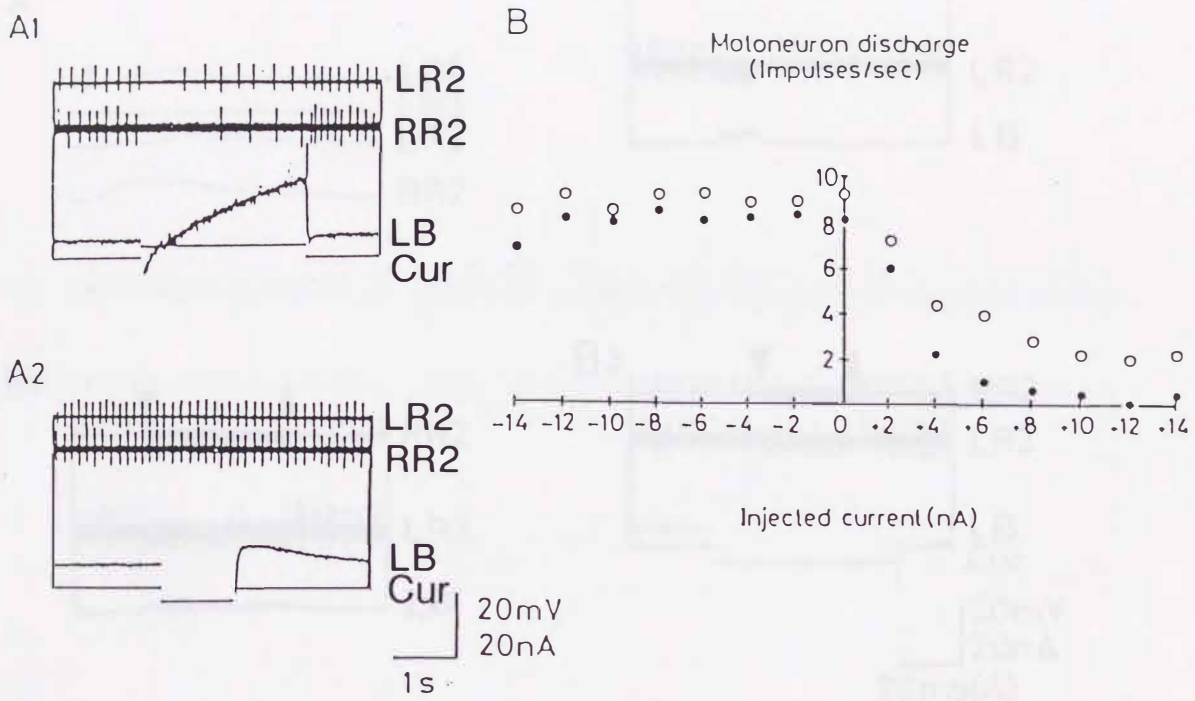

Figure 4. Effect of current injection into LB cell on the spike activity of the second roots. A and B were from different preparations. A1 : injection of 4nA depolarizing current into LB cell inhibited spontaneous spike discharges of the second roots (LR2, RR2). A2: injection of 8nA hyperpolarizing current had no discernible effect on the spontaneous discharge of the roots. B: relation between the intensity of the injected current and spike frequency of the EIs in the right(●) and left (o) second roots.

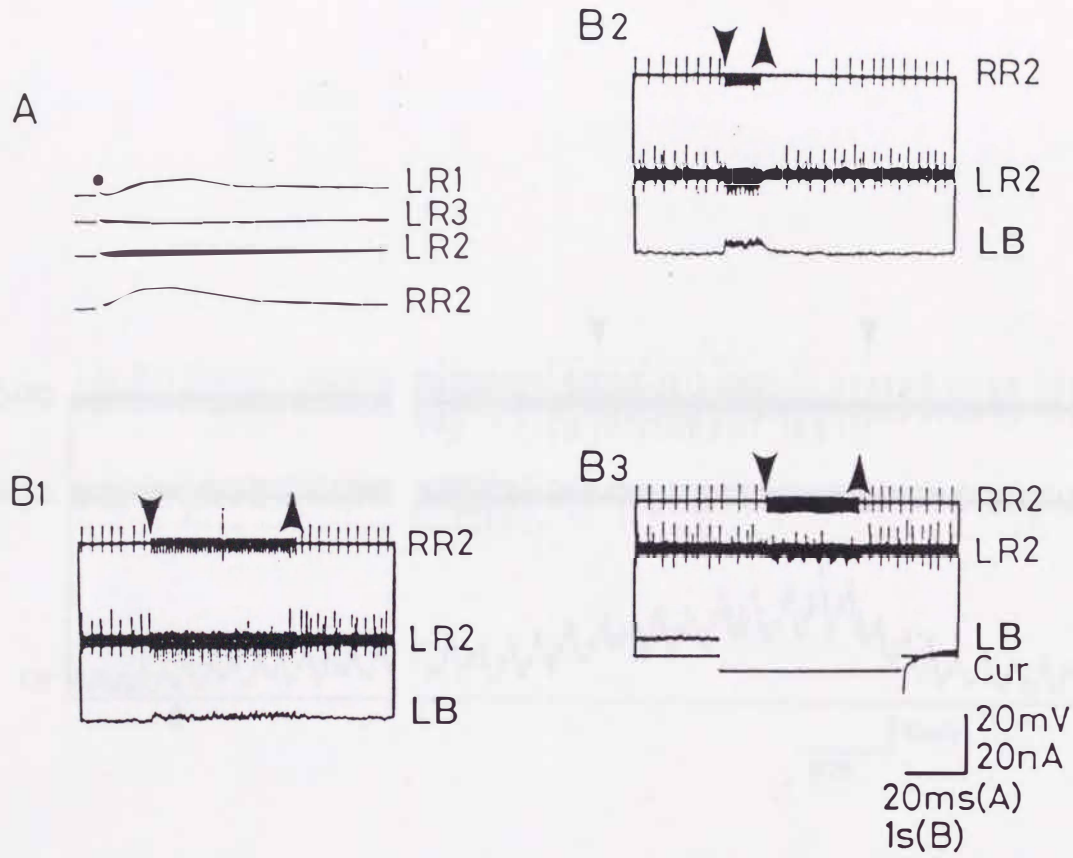

Figure 5. Electrical stimulation of the synaptic pathway to the ganglion. A and B were obtained from different preparations. A: stimulation of nerve roots evoked EPSPs on the LB cell. Each root was stimulated with an electrical pulse of 10 volt, 0.1 ms duration. B: one of the hemiconnective divided into three or four bundles was repetitively stimulated by electrical pulses of 1 volt, 0.1 ms duration. The stimulation inhibited the spontaneous spike discharge of the second roots (RR2, LR2) and evoked PSPs on the LB cell. B1: 20 Hz, 50 stimuli. B2: 80 Hz, 50 stimuli. B3: during intracellular injection of 5nA hyperpolarizing current into the LB cell, the stimulation of 50 Hz, 80 stimuli was applied. Arrowheads show the beginning (▲) and end (▼) of stimulation.

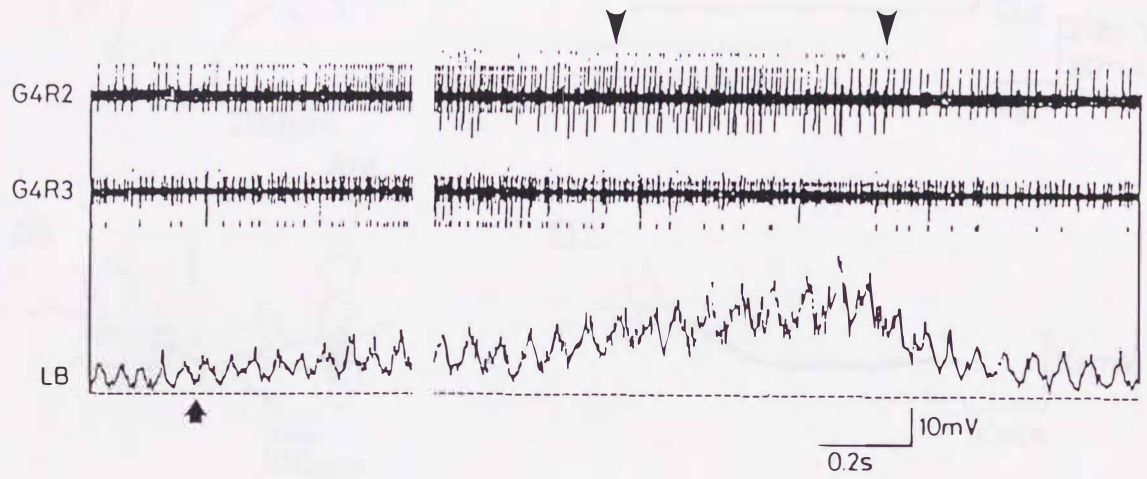

Figure 6. Activity of LB cell during fictive abdominal movement. The animal first showed an increase in the extensor inhibitor (second largest unit in G4R2) and in the flexor excitor activity (G4R3). The LB cell was slightly depolarized when the flexion pattern of motoneuron activity began (indicated by an arrow). A few seconds later, the activity patter of motoneurons changed so that the spike discharge rate of the flexor excitors and the extensor inhibitor then showed a decrease while the largest unit of the extensor excitor in G4R2 showed an increase (arrowheads). This reciprocal pattern of postural motoneuron activities indicated that the network for abdominal extension movement was activated during the period. Correspondingly, the LB cell showed maximal depolarization during the period. After the termination of this fictive movement, the cell repolarized to the previous potential level (as indicated by the broken line). The oscillation in the LB cell's membrane potential (15-20 cycles/sec) seen in this was not observed in other preparations. The functional meaning of this oscillation is not known.

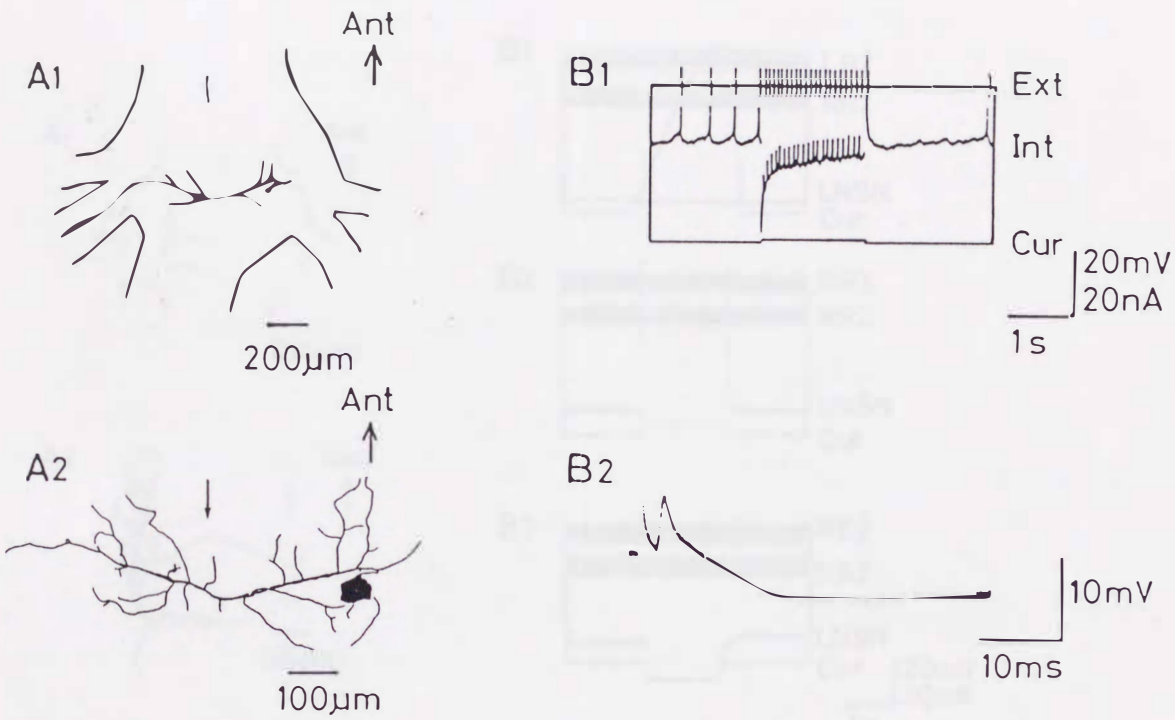

Figure 7. Morphological and physiological identification of the peripheral inhibitory motoneuron innervating the slow extensor muscle. A: morphology of the inhibitory motoneuron stained with Lucifer yellow as viewed ventrally. A1: the location of the neuron in a ganglion. A2: drawing of the reconstructed neuron. Downward arrow indicates midline. B: physiological identification of the neuron. B1: 1:1 correspondence between the extracellular (Ext) and the intracellular (Int) spikes. Intracellular injection of 1nA depolarizing current increased the discharge rate of the spikes in the neuron. B2: antidromic spike induced by stimulation of its axon.

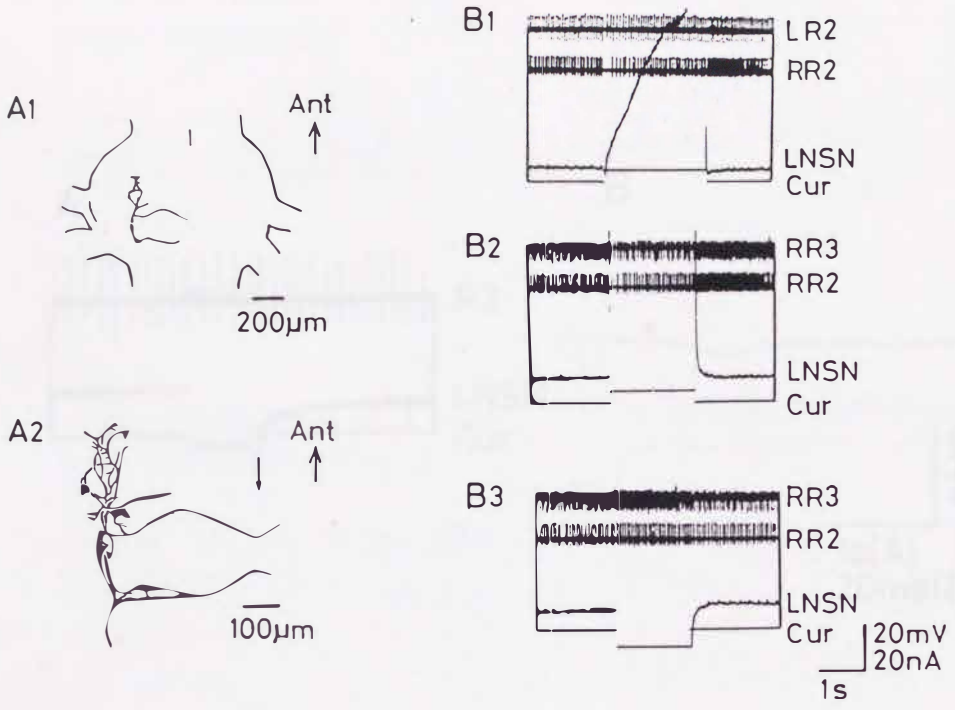

Figure 8. Morphology and physiology of unilateral local nonspiking interneurons (LNSN). A: morphology of the interneuron stained with Lucifer yellow as viewed ventrally. A1: the location of the neuron in a ganglion. A2: drawing of the reconstructed neuron. Downward arrow indicates midline. B1: injection of 5nA depolarizing current inhibited spontaneous spike discharges in both second roots (LR2, RR2). B2: injection of 5nA depolarizing current also inhibited the activity of the third superficial root (RR3). B3: injection of 8nA hyperpolarizing current had no effect on the activity of the both second (RR2) and third (RR3) roots.

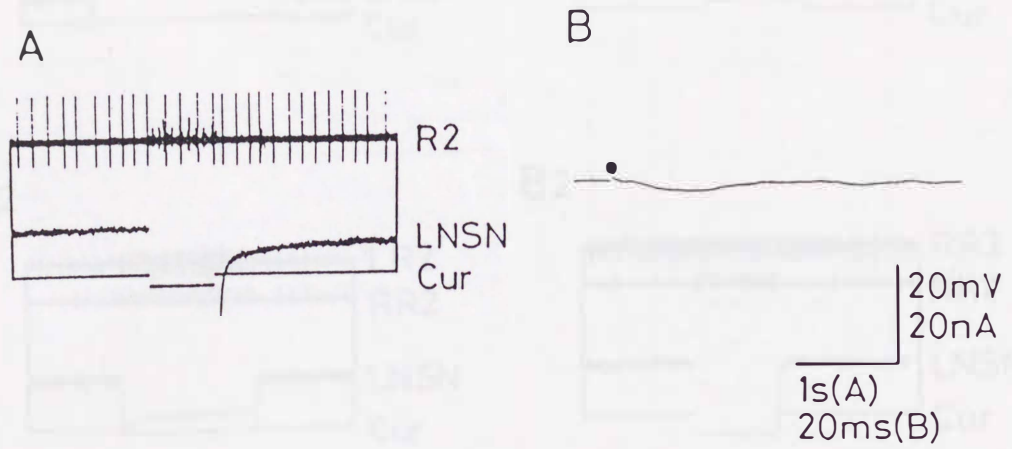

Figure 9. Example of another unilateral local nonspiking interneuron (LNSN). A: injection of 3nA hyperpolarizing current activated several small units in the second root. B: electrical stimulation of the root caused hyperpolarization in the neuron.

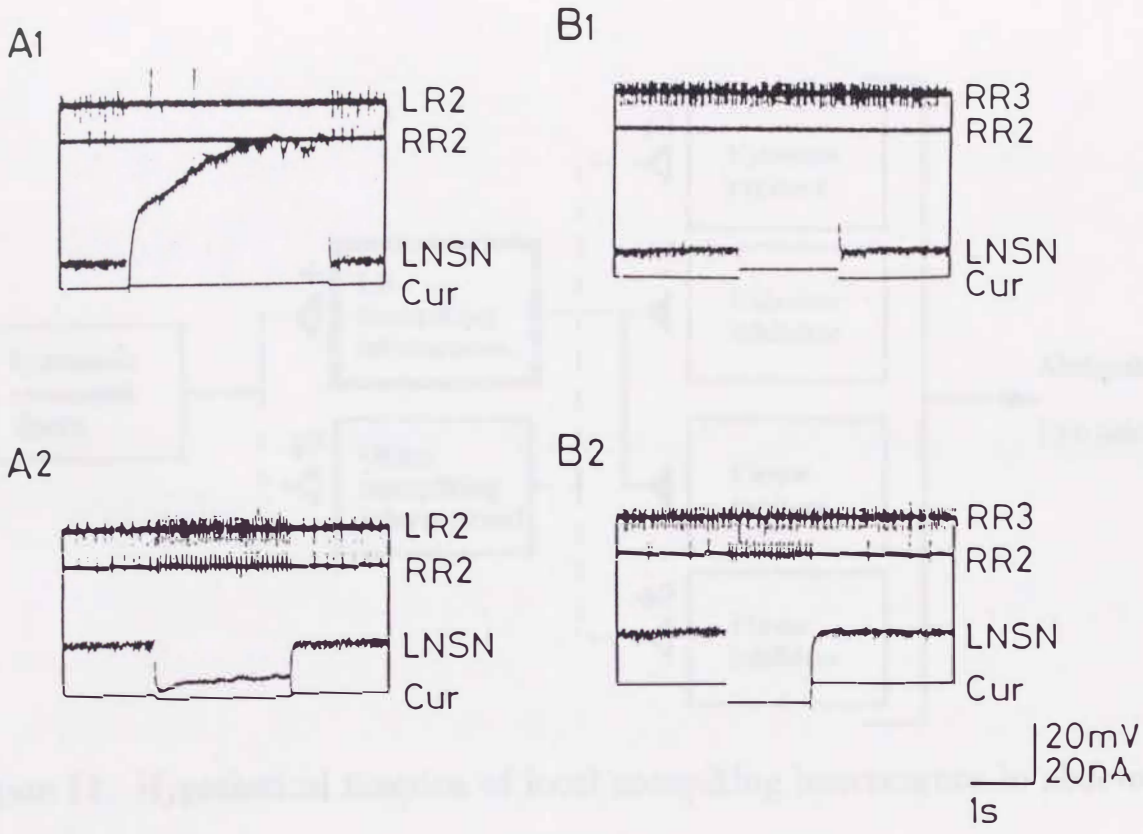

Figure 10. The example of unilateral local nonspiking interneurons (LNSN) in the fifth abdominal ganglion. A1: injection of 1nA depolarizing current inhibited spontaneous discharge of the second roots (LR2, RR2). A2: injection of 1nA hyperpolarizing current activated the spike discharge of the second roots. B1, B2: current injection of either polarity had no effect on the activity of the third roots (RR3).

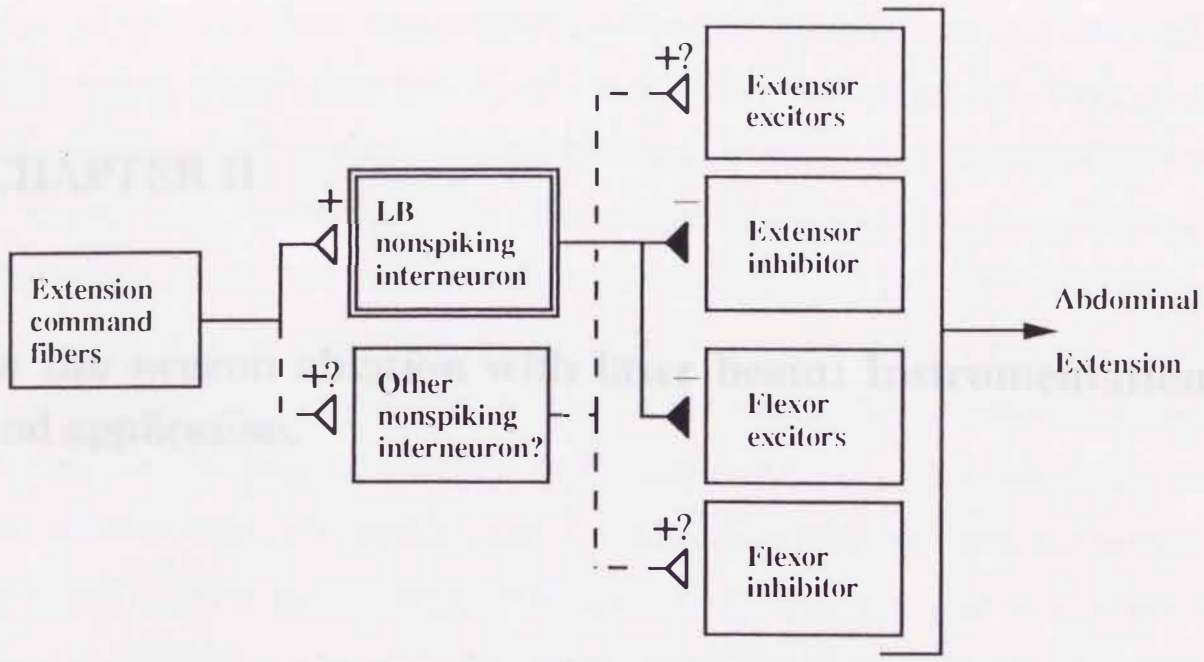

Figure 11. Hypothetical function of local nonspiking interneurons in abdominal posture system.

CHAPTER II

In situ neuron ablation with laser beam: Instrumentation and application.

SUMMARY

Cell killing apparatus was devised by combining a Helium-Cadmium laser with a stereoscopic dissecting microscope. The system can operate as both an epi-illuminating fluorescence microscope and an intense microbeam irradiation apparatus, during electrophysiological recording. A retractable convex lens in the light path can convert the mode of operation of the system. Broader beam illumination by out of focus (flood illumination) was used for in situ observation of fluorescent image and whole cell killing. Intense microbeam irradiation (spot irradiation) was used for partial destruction of a cell. Spot irradiation to the motoneuron axon could sever it by the photodynamic action. As an evidence of the severance, no orthodromic action potential was recorded from the portion peripheral to the irradiated spot, while the rest of the neuron central to it could function normally. The result indicates a possibility that a particular dendritic branch of a neuron could be ablated with this system.

INTRODUCTION

The use of fluorescent dyes for staining a single neuron has been widely employed in morphological analysis of vertebrate as well as invertebrate nervous systems. This technique, especially Lucifer yellow staining (Stewart 1978), has been used in a variety of neurophysiological approaches: *in vitro* visualization (Stewart 1978); *in vivo* visualization (e.g. Reichert and Krenz 1986); killing of neurons or a part of neurons (e.g. Miller and Selverston 1979; Jacobs and Miller 1985).

Cell killing by photodynamic action of fluorescent dye was first made practical by Miller and Selverston (1979). They developed a method for a selective neuron destruction by combined use of intracellular staining with Lucifer yellow and illumination by high intensity blue light. Using this technique of photoinactivation, they judged the role and the relative importance of several functional components of the lobster pyloric system (Selverston and Miller 1980). Since then there have been several other studies using the photoinactivation in neurophysiological and behavioral analysis: cricket cercal sensory system (Jacobs et al. 1986); cricket auditory system and phonotaxis (Selverston et al. 1985; Atkins et al.,1984); cockroach escape behavior (Libersat et al. 1989). All these experiments utilized a combination of the intracellular Lucifer injection and the illumination with intense short wavelength (blue) light.

Several ideas of constructing the illumination apparatus for *in situ* dye visualization have been reported recently (Kater et al. 1986; Reichert and Krenz 1986; Heitler and Fraser 1989). In this chapter, I report a new epi-illumination microscope system which enables us to perform a quick observation of cell structure and subsequent identification *in situ* of crayfish neuron. The system is also convertible into a cell killing microbeam irradiator by a simple adjustment of its optics. I tested the whole cell inactivation by flood laser light epi-illumination, and then made a partial destruction of a neuron by spot epi-illumination.

MATERIALS and METHODS

I adopted a Helium-Cadmium laser (Omnichrom Co.Ltd., model 456-30MS, California, U.S.A.) for the dye stimulation. Schematic arrangement of the system is shown in Fig.1. Laser beam was focused on the input end of a quartz fiber light guide (Fujikura Densen Co.Ltd., model GC 50/125, Tokyo, Japan) through a focusing lens. A neutral density filter mount and an electrical shutter were placed between the laser and the lens. The output end of the fiber was mounted on the epi-illuminator of the stereoscopic dissecting microscope of a long working-distance (200mm) (Olympus Co.Ltd., model MTX, Tokyo, Japan). The conventional illuminator originally provided was modified so as to attach the fiber and suit to pass the laser light. The illuminator focused the laser beam onto the preparation.

A convex lens was inserted in the optical pathway of the laser beam. This lens, which is readily removable from the optical pathway, brings the beam of laser light out of focus on the preparation giving a broader and more diffuse illumination suitable for observation of fluorescent image of the stained cell.

All experiments were carried out on adult crayfish *Procambarus clarkii* Girard of both sexes. The crayfish had a body length of 8-11 cm from rostrum to telson. They were obtained commercially (see chapter I). The abdominal nerve cord was isolated and pinned dorsal side up on a Sylgard-lined dish filled with crayfish saline (van Harreveld 1936). Suction electrodes were used for extracellular recording of motoneuron activity and nerve stimulation. A glass capillary microelectrode was used for intracellular recording and dye injection. Tips of the microelectrodes were filled with the dye solution of 5% Lucifer yellow CH in 1 M $LiCl_2$ (Stewart 1978). Electrode resistances were 30-60 M Ohm. The dye was injected iontophoretically with pulsed hyperpolarizing current of 5nA, for 20 minutes.

RESULTS and DISCUSSION

1. Laser beam illumination, mode of operation

The converted stereoscopic dissecting microscope of a long working-distance kept wide space for electrodes setting between the microscope and the preparation. The system, therefore, allowed electrophysiological recording by micromanipulated intracellular electrode under the microscopic observation with ordinary external light source. Diffused laser light illumination for cell identification and focused irradiation for photoinactivation were done through the epi-illumination installation of the laser light beam.

The optimal stimulating wavelength of Lucifer yellow CH is 430 nm (Stewart 1978). The Helium-Cadmium laser has the wavelength of 441.6 nm, which was found to be still appropriate for the dye stimulation and for the observation of fluorescent image of the stained cell. To cut the laser light off from the observing image, a yellow filter of JIS SY-48 was placed before the objective lenses of the microscope. This filter is a low-pass filter and has 50% transmittance wavelength at 480 nm. Since Lucifer yellow CH has maximum emission wavelength at 540 nm (Stewart 1978), this filter could effectively cut off the reflection of blue light from the preparation and the saline surface and allow the emission to pass through for observation.

The laser beam was focused into a 40 micrometer in diameter circular spot confocal with the focal plane of the microscope (spot irradiation). This mode of irradiation was used for partial ablation of a neurite of a single neuron. Insertion of a small convex lens into the optical pathway gave a broader illumination suitable for observation of fluorescent image of the cell or whole cell killing (flood illumination). In both cases, the laser light has a incident angle tilted 5.4^{o} off from the optical axis of the microscope. This inclination prevent the direct reflection of laser beam from the saline or preparation surface from coming into the objective lens. An iris and a ring made of a orange colored acrylic were placed in the

optical pathway to prevent and absorb the internal flare by spurious reflection of laser beam.

The output end of the optic fiber is held by X-Y stage attached on the epi-illuminator of the dissecting microscope. Adjusting the stage, the fiber end shifts its position around the optic axis. Since the position determines the target location of the beam in the microscope viewing field, the laser beam can be placed at a certain point (usually at the center) of the viewing field by adjusting the stage in the mode of spot irradiation. Integrated unitary construction of the laser optics and the dissecting microscope made the determination of the target location very simple to perform.

The newly devised system thus could work both as an epi-illuminating fluorescence microscope and as an intense microbeam irradiation apparatus. An example of *in situ* visualization of a neuron and the electrode penetrating it is shown in Fig.2. *In situ* visualization of electrode penetration site on a neuron is quite useful to facilitate functional analysis of the local property of the penetrated part of the neuron such as the identification of actual synaptic site of dendro-dendritic connection among motoneurons which has been electrophysiologically postulated by Nagayama et al (1983). Prolonged flood illumination will result in the whole cell killing, which is also useful to determine the role and the relative importance of specific identified neurons in a neural network (Selverston and Miller 1980).

Spot irradiation was used for the partial destruction of a dye filled neuron (Jacobs and Miller 1985). This application is to obtain information pertinent with the role of the ablated part of the neuron. Examples of this line of study will be given in the following section.

2. Whole cell inactivation by flood laser light epi-illumination

A tonically discharging motoneuron was penetrated in the fourth abdominal ganglion. The motoneuron was identified as a slow extensor inhibitory

motoneuron (EI) by physiological and morphological features of it (Wine and Hagiwara 1977; Miall and Larimer 1982; see also chapter I). Before the laser epiillumination, the motoneuron showed spontaneous discharge of 0.6 spikes/sec, and its resting potential was -63 mV. The spontaneous spike discharge was used as a criterion of the motoneuron activity. After the microelectrode penetration into the motoneuron, the cell was iontophoretically filled with Lucifer yellow, and the ganglion was continuously epi-illuminated by flood laser light through the apparatus.

Figure 3 shows the result of the illumination. After the illumination began, spontaneous spike discharge rate of the motoneuron gradually increased. Resting potential of the motoneuron also gradually depolarized. In the course of the illumination, the resting potential showed a fluctuation of repetitive depolarization and repolarization. The resting potential temporarily changed to depolarization level, and then repolarized to previous potential level. Difference of the potential level between the resting and the depolarization was approximately 20 mV. The fluctuation lasted about 10 seconds, and then did not return to the initial level. After the termination of the fluctuation, resting potential of the motoneuron further showed gradual depolarization. Spontaneous spike discharge also lasted after the termination of the fluctuation for about 1 minute, and then ceased abruptly (Fig.3D). After the ceasing of the spontaneous discharge, discharge of the neuron was no longer observed. Since the spontaneous discharge did not recover, laser epi-illumination was terminated. Total duration of this illumination was three minutes. The motoneuron showed its membrane depolarization of 34 mV after the end of the illumination.

Selverston and Miller (1980) represented three criteria to judge the neuron photoinactivation. The results of the experiment corresponded with two in the criteria of ceasing action potential in the cell's peripheral axon, and drastic depolarization of the cell. Therefore, I judged that the illuminated motoneuron was photodynamically destructed. No increase of firing rate of other motoneurons

projecting their axons in recorded nerve root indicates that these unstained neurons were still intact after the illumination.

3. Axonal ablation with laser photoinactivation

Axonal ablation was tried on the slow extensor inhibitory motoneuron in the fourth abdominal ganglion. The neuron was first identified with dye filling and subsequent *in situ* visualization of its morphology by the flood illumination with the apparatus (Fig.4A). EIs exist as a bilateral pair of motoneurons in the ganglion. They are also characterized with spontaneous firing and functional coupling between them. Injection of intracellular current into one motoneuron changed spike activity of the contralateral homologue (Fig. 4B).

The laser beam by spot irradiation was aimed at the dye filled axon near the root exit. The irradiated part was located between the intracellular electrode in the ganglion and the extracellular electrode on the root. In the case shown in Fig.4, the axon was repeatedly irradiated with 15 seconds exposure. The irradiated neuron increased its spontaneous firing rate in the course of irradiation (Fig.3C). After 1 minute of cumulative irradiation time, orthodromic action potential of the neuron was no more recorded with the extracellular electrode which was distally placed beyond the irradiated part. Spontaneous spike generation, however, was recorded with the intracellular electrode. Fig.4D shows the result after 2 minutes irradiation. Intracellular injection of hyperpolarizing current still inhibited the spontaneous firing of the contralateral homologue (Fig.4D). After the illumination axonal bleaching was found at illuminated region (Fig.4E). Consequently, the motoneuron has yet kept the ability of its own spike generation and the functional coupling with its contralateral homologue in the ganglion, after the axotomy by photoinactivation. The continuance of the coupling indicates that EIs motoneurons have the sites of local interaction in the central neuropil. The motoneurons could have dendro-dendritic interactions between them as well as to the uropod closer motoneurons in the sixth abdominal ganglion (Nagayama et al.

1983). This kind of interaction between motoneurons would exist among other motoneurons located in the ganglion.

We concluded that the inactivation induced by spot irradiation was limited to the irradiated region. The limited inactivation can thus ablate particular dendrites from a crayfish neuron, as shown in cricket cercal interneuron (Jacobs and Miller 1985; Jacobs et al. 1986). By performing similar experiments in which the laser beam was aimed at different parts of the dendritic tree of a neuron, we will be able to assess the relation between neuronal structure and function. Pearson (1979) proposed that the different arborizing regions of invertebrate neuron are electrically isolated from each other and therefore function more or less independently. If the function of a certain dendrite of a neuron was studied by the dendrite ablation with the current method, Pearson's hypothesis could be practically tested. Also, the response properties of various neurons could be interpreted in terms of synaptic interaction at single dendrites. In addition, in situ visualization of neuronal structure enables us to accomplish multiple electrode penetration into a single neuron under visual control (Reichert and Krenz 1986). The multiple penetration is useful and necessary to study passive properties of a single neuron in the central nervous system.

Besides the neurophysiological application of the current method, principle of the photoinactivation and *in situ* visualization will be utilizable in other fields of biology. Some possible applications are: tracing of a cell-lineage by fluorescent dye injection into a cell and subsequent laser killing of the cells belonging to the line to know the developmental fate and the role of the cells in an embryo; assessment of the functional role of a specific cell group in development by selective laser killing; visualization of morphological change of living cells during pre- and post- embryonic development. This laser apparatus thus may serve as a new versatile tool in biology.

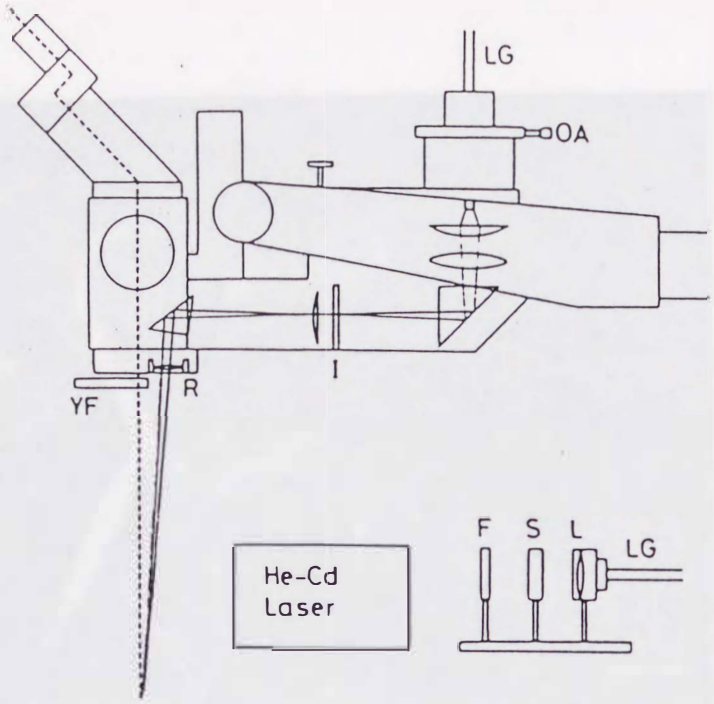

Figure 1. Schematic diagram of the laser light epiillumination apparatus. Laser light from the generator was focused by a lens (L) and guided to the dissecting microscope through a quartz fiber light guide (LG). An electrical shutter (S) and a neutral density filter (F) for light intensity control were interposed between the generator and the input end of the light guide. An iris (I) and a ring (R) to prevent and absorb internal flare were also inserted in the optical pathway. OA: X-Y stage adjuster holding output end of the light guide. YF: yellow filter for eye protection. This microscope was used for both laser light epi-illumination and observation of preparation.

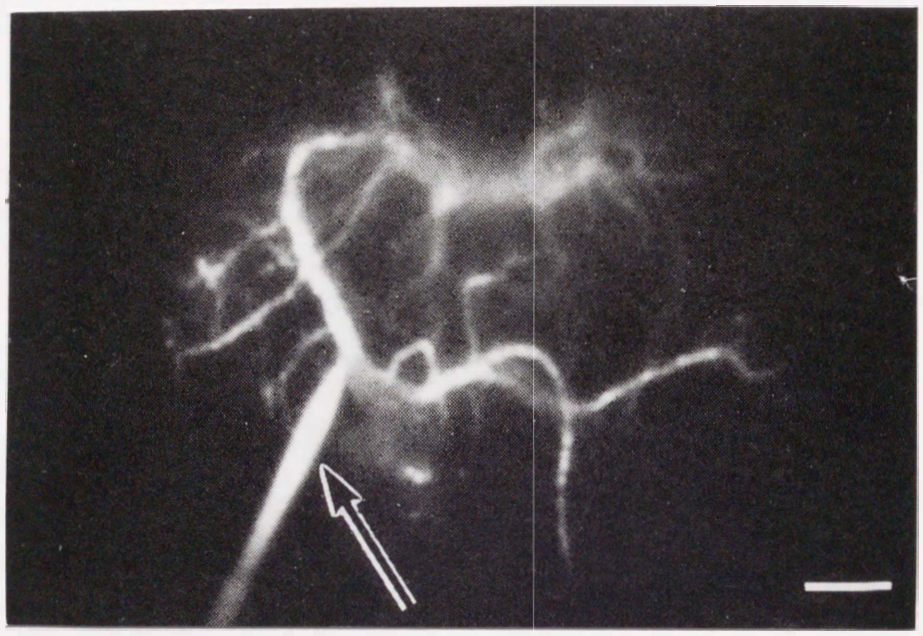

Figure 2. Photograph taken through the dissecting microscope. This photograph shows the structure of a dye filled neuron in the crayfish sixth abdominal ganglion and a microelectrode penetrating it. The neuron viewed dorsally. An arrow indicates the electrode. Arrowhead indicates soma of the neuron. Anterior is at the top. Calibratiob bar: 100μm

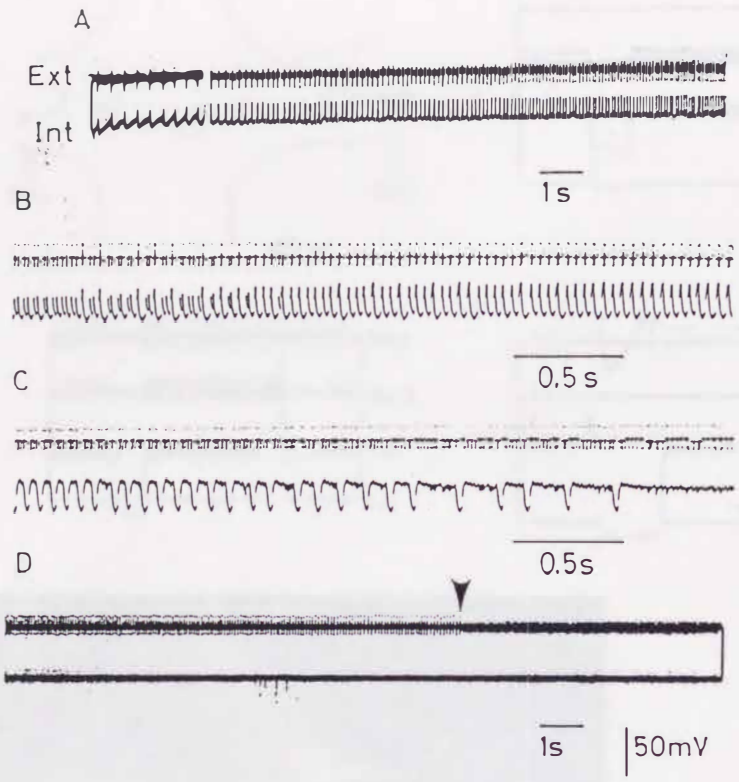

Figure 3. Spontaneous spike activity of a slow extensor inhibitory motoneurons in the fourth abdominal ganglion during flood laser light epiillumination. A: spike frequency of the dye injected and irradiated motoneuron gradually increased. B, C,: the cell shows fluctuation of its resting potential level. D: termination of the spontaneous spike discharge. Arrowhead shows the last spike discharge of the motoneuron. Abbreviations: Ext, extracellular recording from the axon of the motoneuron. Int, intracellular recording from a dendrite of the motoneuron.

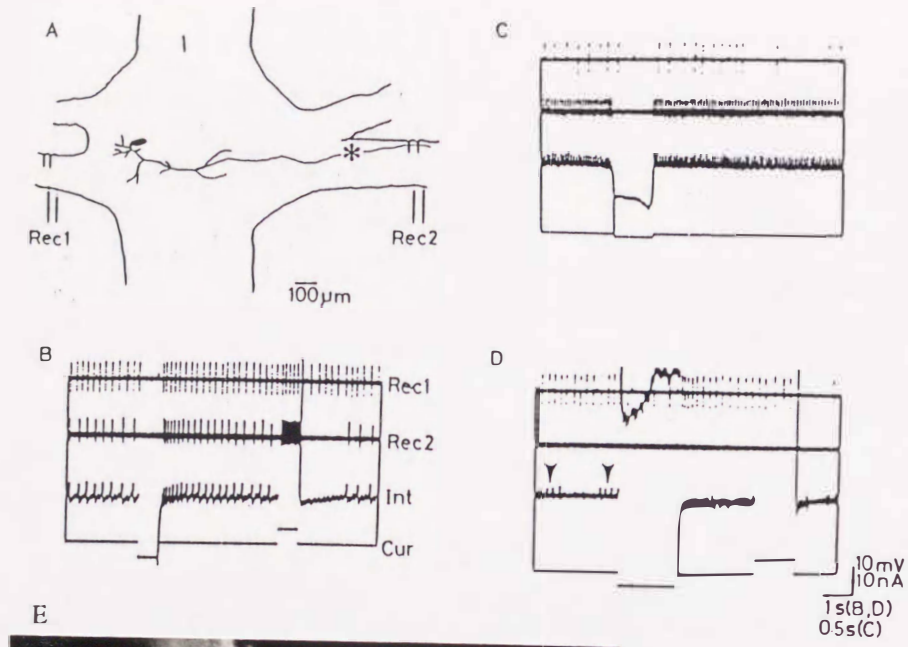

Figure 4. Effect of axonal ablation on the activity of the slow extensor inhibitory motoneurons in the crayfish fourth abdominal ganglion. A: experimental setting. A solid line drawing shows dye injected and irradiated motoneuron, and a broken line drawing is contralateral homologue of the motoneuron. Asterisk indicates irradiated portion of laser beam. The neurons viewed ventrally. Anterior is at the top. B: spike activity of the motoneurons before irradiation. Intracellular injection of 5nA current of either polarity into the stained motoneuron (3rd trace) changed its own extracellular spike activity (2nd trace) and that of contralateral homologue (1st trace). C: spike activity of the neurons after for 45 seconds irradiation. Spike activity of the irradiated neuron increased. Injection of -1nA current into the

irradiated neuron effected its homologue. D: the result after the irradiation for 2 minutes. Extracellular spikes of the irradiated neuron disappeared (2nd trace), but motor output to the contralateral homologue (1st trace) and intracellular spikes of the neuron (arrowheads on 3rd trace) still remained. E: photograph of the neuron after the irradiation. Arrow indicates the irradiated portion.

CHAPTER III

Cross connections coordinate postural motoneuron activity in the crayfish abdomen.

SUMMARY

Intracellular recordings and dye injections were used to examine mutual coupling among slow abdominal postural motoneurons in the fourth abdominal ganglion in crayfish. Intracellular current injection into one motoneuron altered the spike firing rate of some its synergists. Depending on the polarity of the injected current, the premotor effect on the synergists was excitatory or inhibitory. The magnitude of the effect was intensity dependent. No dye coupling was found among the motoneurons following injection of Lucifer yellow. The morphological basis of the coupling was examined by differential filling of motoneurons pairs, one with horseradish peroxidase and the other with Lucifer yellow. The stained motoneurons were simultaneously visualized under light microscopy to determine the proximity of their differently colored dendrites. It was thus possible to locate the site of the presumed monosynaptic contacts between them. Combined physiological and morphological evidence suggests that these neurons are mutually coupled. Consequently, the coupled motoneurons form part of an integrative system for abdominal posture control in crayfish.

INTRODUCTION

The crayfish abdominal posture control system has been studied as an example of a neural circuit underlying a simple behavior. In this system, single interganglionic interneurons dissected out from the abdominal nerve cord could cause a coordinated motor pattern for abdominal posture movement when they were activated (Kennedy et al. 1966b). The interneurons that initiate abdominal flexion and extension movement have been called "command fibers" after the terminology introduced by Wiersma and Ikeda (1964) for swimmeret movements in crayfish (see also Kennedy et al. 1966b; Evoy and Kennedy 1967). According to the command fiber hypothesis, the efferent neurons have been considered as forming a passive pathway to conduct neural signals from command fibers to postural muscles. The coordination of all except the largest efferent neurons controlling the posture of the crayfish abdomen is dependent on connections organized at a premotoneuron level (Tatton and Sokolove 1975), proximal to the motoneurons (Kennedy 1969).

Spike train analysis by Tatton and Sokolove (1975) showed that the larger postural motoneurons had both excitatory and inhibitory connections among them, and that the connections partially contributed to the neuronal coordination for postural movement, though the authors did not succeeded in making intracellular recordings from the motoneurons. They emphasized the significance of the connections for achieving postural control. Miall and Larimer (1982) showed some examples of weak coupling between slow flexor motoneurons by current injection experiments. I have preliminary showed bilateral coupling between the slow extensor inhibitory motoneurons (EI) by intracellular recording and laser photoablation. The functional significance of cross coupling among motoneurons was also reported in other crayfish motor control systems (swimmeret, Heitler 1978; uropod, Nagayama et al. 1983; walking leg, Chrachri and Clarac 1989). Further examination is necessary to elucidate the contribution of the coupling phenomenon to the abdominal posture system.

The slow flexor motoneurons extend their neurites on both the ipsilateral and contralateral sides of the ganglion to their somata. Their dendritic domains occupy similar areas in the ganglion (Wine et al. 1974; Leise et al. 1986). Wine et al. (1974) suggested that closely located neurites between mirror-imaged contralateral homologues provided the morphological substrate for the motoneuronal connections that had been demonstrated by Tatton and Sokolove (1975). Their suggestion was not based on differential filling of a set of motoneurons, but on axonal retrograde fillings of plural motoneurons by cobalt dye. With this procedure, it is not possible to fill individual neurons selectively and to identify actual contact between a pair of neurons. Differential cell marking by intracellular dye injection would give direct evidence of the possible contact of neurites between contralaterally homologous flexor motoneurons.

In this chapter, I will demonstrate functional coupling among abdominal postural motoneurons in crayfish using intracellular recordings and a stimulation technique. The morphological substrate for the physiologically observed coupling was also obtained under light microscopy.

MATERIALS and METHODS

1. Animals and Preparation

Adult crayfish, *Procambarus clarkii* Girard, of either sex, measuring 8–11 cm from rostrum to telson, were obtained commercially and kept in laboratory tanks before use.

I used two kinds of preparations for the experiments. The first type, an isolated nerve cord preparation, was used for recording of the activity of the slow extensor motoneurons in the fourth abdominal ganglion (see chapter I). The abdominal nerve cord was dissected out of the abdomen and pinned dorsal side up in a silicon rubber lined dish filled with crayfish saline (Van Harreveld 1936). The second type of preparation was an isolated abdomen, and was used for recordings of the slow flexor motoneuron activity. The abdomen was isolated from the rest of the body, and pinned ventral side up in a dish filled with the same saline. The fourth ganglion was exposed and stabilized on a stainless plate.

2. Extra- and intra-cellular recordings and stimulation

Spike activity of the abdominal postural motoneurons was monitored extracellularly from their axons by means of suction electrodes. The electrodes were placed on the second roots and on the superficial third roots of the fourth abdominal ganglion. The slow flexor motoneurons in the ganglion were artificially activated by applying electrical stimulation to afferent axons in the fourth root of the sixth abdominal ganglion (Tatton and Sokolove 1975).

A glass capillary microelectrode for intracellular recording was driven across the sheath of the ganglion. The ganglionic sheath was treated with protease (Sigma: Type XIV) for 2–5 min before electrode penetration. In many preparations, the electrode was inserted into the soma of a slow flexor motoneuron from the ventral side. In contrast to that of the flexor, the electrode was inserted into the large dorsal process of the slow extensor motoneuron near the midline in the ganglion from the dorsal side. The electrodes contained a dye solution of 3% Lucifer yellow (DC resistances of 50–100 MOhm; Stewart 1978) or of 4%

horseradish peroxidase (DC resistances of 20–50 MOhm). Occasionally, a dye solution of 5% 5(6)-carboxyfluorescein (Kodak) in 0.44 M KOH was used (DC resistances of 30–50 MOhm; Purves et al. 1986). The current was injected into the cell through a microelectrode via a bridge circuit. The motoneurons were identified physiologically both by checking the one-to-one correspondence between the intra- and the extracellular spikes and by antidromic stimulation of their axons.

3. Intracellular staining and simultaneous visualization of two motoneurons

Following the intracellular recording, the motoneuron was stained by dye injection. To observe the morphological basis of the coupling between flexor motoneurons, we adopted and modified the differential staining technique of the combined use of horseradish peroxidase and Lucifer yellow (Macagno et al. 1981; DeRiemer and Macagno 1981).

The electrodes for horseradish peroxidase (HRP) injection were filled with a 4% solution of HRP (Sigma: Type VI) in Tris buffer (pH 7.4) containing 0.5M KCl. HRP was injected into a motoneuron iontophoretically by applying half-duty cycle depolarizing current pulses of 1Hz and 20–30nA for 30–60 min. During HRP diffusion, the second motoneuron was filled with Lucifer yellow (3% in 0.1M LiCl2; Stewart 1978) using hyperpolarizing current pulses of the same frequency and 5–10nA for 1hr. Following the Lucifer injection, the ganglionic sheath was partially removed and HRP was developed by the method of Macagno et al. (1981). Following a 30 min–1 hr fixation with 10% formalin in crayfish saline, the ganglion was dehydrated in graded ethanol washes, then cleared and mounted in methyl salicylate.

The dye-filled motoneurons of fluorescent and opaque images were observed and photographed using transmitted fluorescence microscopy. The observation was best achieved by placing a deep-blue filter in front of the fluorescence light source. After whole mount observation, the ganglion was immersed in benzene for 15 min and embedded in paraffin wax. Serial sections 10

μm in thickness were cut, observed and photographed under the same condition as that of the whole mount observation.

RESULTS

1. Connection between slow extensor inhibitory motoneurons

The second root of from the first to the fifth abdominal ganglia contains the axons of the slow extensor motoneurons (Kennedy et al. 1966a). In preparation of an isolated nerve cord, only one unit with the large spike amplitude usually showed spontaneous firing (2-9 spikes/sec) in the extracellular recording. Lucifer staining of the spontaneous unit revealed the soma contralateral to the axon's exit, and the bilateral distribution of its neurites in both hemiganglia. The soma was about 50 μm in diameter (Fig.1A). These morphological features corresponded with that of the slow extensor inhibitory motoneuron which innervates the slow extensor muscle (Wine and Hagiwara 1977; Miall and Larimer 1982; see also chapter I).

Intracellular current injection into the extensor inhibitory motoneuron (EI) altered the spike frequency of the contralaterally homologous EI (n=9). Injection of a depolarizing current into the EI increased the spike firing rate of EIs in both hemiganglia (Fig.1B). Injection of a hyperpolarizing current conversely decreased their firing rates (Fig.1C). EI exerted a bi-directional premotor effect on its contralateral homologue. The direction of the premotor effect depended on the polarity of the injected current. The magnitude of the effect was intensity dependent (Fig.1D).

An injection of current into EI showed no discernible effect on the spike activity of other units in the pool of the slow extensor motoneurons except the contralateral EI. There was no dye-coupling between EIs following the Lucifer injection. I examined connectivity between EI and flexor motoneurons in two preparations. In one preparation, spontaneous spike discharge of the contralateral superficial third root was inhibited by a depolarizing current injection into the EI (Fig.2A). The magnitude of the inhibition depended on the intensity of the current. Injection of a hyperpolarizing current, however, had no effect on the spike activity of the third root units (Fig.2B). The inhibited units seemed to be slow flexor

excitatory motoneurons because of their tonic discharging and their middle-sized spike amplitude (Kennedy and Takeda 1965). No discernible connection was detected in the other preparation.

2. Connections among slow flexor motoneurons

The abdominal flexor muscle in each hemisegment is controlled by six slow flexor motoneurons that have been numbered sequentially according to increasing spike heights as recorded in the third root (Kennedy and Takeda 1965; Wine et al. 1974). I recorded intracellularly from the slow flexor excitatory motoneurons No.4 (F4) and No.6 (F6). These two motoneurons were identified by the following four features: extracellular spike amplitude, postero-lateral soma position, ipsilateral axon to its soma and gross morphology. These features corresponded with those cited in previous reports on F4 and F6 (Wine et al. 1974; Miall and Larimer 1982).

Current injection into F4 altered spike frequency of tonically discharging units of middle-sized amplitude in the contralateral third root (n=10). Injection of a depolarizing current increased the spike firing rate of the units (Fig.3B). Injection of a hyperpolarizing current had an opposite effect on the units (Fig.3C). In the extracellular recording, F3 and F4 showed middle-sized spikes of similar amplitudes, and could not be reliably separated by either spike height or firing pattern (Kennedy and Takeda 1965; Wine et al. 1974). The modulated units may be composed of F3 and F4. The effects of current injection on the units were related to the intensity of the depolarizing and hyperpolarizing current (Fig.3D).

F4 also exerted premotor effect on F6s in both hemiganglia (n=17). The injection of a depolarizing current into F4 raised the spike firing rate of F6s (Fig.4A), and the injection of a hyperpolarizing current depressed the rate (Fig.4B). During the artificial activation of F6s by the electrical stimulation applied to the fourth root of the sixth ganglion (see MATERIALS and METHODS), a current injection into F4 either facilitated or canceled the spike firing of F6s (Fig.4C). The cancellation effect suggests a predominance of the connection among the

motoneurons over the intersegmental input from the caudal ganglion. The magnitude of the premotor effect on F6s depended on the intensity of the injected current (Fig.4D). F4 had no premotor effect on the slow flexor inhibitory motoneuron in the ganglion.

The injection of a depolarizing current into F6 raised the spike firing rate of the middle-sized units of F3 and F4 (n=8), and also of the contralateral F6 (n=19). The injection of a hyperpolarizing current depressed their spike firing (Fig.5B-D). Hyperpolarization of F6 could cancel the effect of the artificial activation of the contralateral homologue by stimulation of the fourth root of the sixth ganglion. The connection between F6s was more dominant than the connection between F6 and the ascending sensory pathway from the caudal ganglion. The spike frequency of the contralateral F6 was altered depending on the polarity and the intensity of the injected current (Fig.5D). No dye-coupling was found among the slow flexor motoneurons by the Lucifer injection.

3. Simultaneous visualization of two different motoneurons

The morphological substrate of the coupling between flexor motoneurons was examined by differential filling of pairs of motoneurons by horseradish peroxidase (HRP) and Lucifer yellow (LY) under fluorescent microscopy. The image was darker in the neuron filled with HRP and was lighter in that with LY.

A set of F4s was differentially stained and simultaneously observed. F4s have extensive areas of overlap of their dendrites in both hemiganglia. Each neuron sends a large dorsal process across the midline to the contralateral margin of the neuropile. The process of about a 6 μm diameter was in close proximity with that of its contralateral homologue (Fig.6A). Transverse sections were cut through the ganglion to observe the detailed relation between the processes and the ganglionic structure. These processes were located in dorsal commissure 3 (DC3; Skinner 1985), and were seen to lay adjacent for some of their length in the commissure (Fig.6B1).

F4s give off numerous dendrites at both lateral margins of the ganglion. Bilaterally extended dendrites of different colors of F4s occupied similar areas in the ganglion and showed close proximity between them (Fig.6A). The close proximity of the dendrites was mainly observed in the posterior region of the dorsal intermediate tracts (DIT; Skinner 1985), though it was widely distributed in the ganglion (Fig. 6B2).

F6 also has a bilateral structure of its dendrites. Differential staining of F6s revealed an overlap of their dendritic domains (Fig.7A). A dorsal process that crossed the midline was in close proximity with that of its contralateral homologue. Processes of about 6 μm in diameter lay in dorsal commissure 3 and had close contact with each other (Fig.7B1). Their differently colored dendrites were also in close proximity in the ganglion (Fig.7A, B). Similar apparent contact was also observed between F4 and F6 (Fig.8).

DISCUSSION

This study demonstrates that several abdominal postural motoneurons are coupled to each other to function as integrative elements in the CNS for abdominal flexion movement. A motoneuron could synergically alter the spike activity of other motoneurons depending on its own activity as induced by an intracellular injection of current. This intracellular study confirms the results of previous study shown by Tatton and Sokolove (1975) with extracellular spike analysis. In addition to the examples of coupling demonstrated by Tatton and Sokolove (1975), I found other examples of coupling (Table 1). The identified coupling, EI coupling and flexor couplings, were consistent with the functional mode operation of the neurons in the postural movement. The inhibitory connection between EI and the flexor excitatory motoneurons, however, contradicts the mode of operation for flexion movement. This was an exception of the functional coupling.

Slow flexor muscles are located in the right and left halves of each abdominal segment. The musculature in both hemisegments contracted bilaterally by stimulation of a command fiber (Kennedy et al. 1966a; Evoy and Kennedy 1967). Evoy and Kennedy (1967) hypothesized that coordination for the bilateral contraction was due to the existence of some ganglionic driver interneurons which received input from command fibers and, in turn, distributed the input bilaterally to the motoneurons. There is, however, no report of the existence of the driver interneurons involved in abdominal flexion movement. It has been indicated that the bilateral coordination for abdominal positioning movements might be achieved by a ganglionic driver circuit constructed from several abdominal positioning interneurons, which had been through as command fibers (Larimer 1988; Murphy et al. 1989). In the circuit, each of the interneurons contributed to a small part of the overall motor output (Murphy et al. 1989). Coupled motoneurons may participate in the circuit to guarantee bilateral operation of a set of flexor muscles located in each hemisegment. However, it should be noted that highly effective

coupling reduce the repertoire of movements available to a given motor system (Tatton and Sokolove 1975).

The sheet of a slow flexor muscle is under polyinnervation by flexor motoneurons (Kennedy and Takeda 1965). Simultaneous activation of plural motoneurons by stimulation of command fibers produced a smoothly rapid rise and static maintenance of muscle tension, while the tension developed by a single motoneuron showed a slow rise and often failed to reach its maximum plateau (Evoy and Kennedy 1967). Mutual couplings among the motoneurons are suitable for producing rapid alteration in muscle tension (Sokolove and Tatton 1975; Tatton and Sokolove 1975). The coupling, especially ipsilateral F4-F6 coupling, may contribute to adjustment of the development of tension through the simultaneous activation of motoneurons in a given flexor muscle sheet.

In some preparations, flexor motoneurons showed no discernible coupling with their synergists throughout our experiment (n=26). This might suggest less of a contribution of the coupling in the ganglionic driver circuit in producing the flexion movement. However, I cannot eliminate the possibility that these ineffective 26 cells could exert any premotor effect on their synergists. The ineffectiveness of the current injection was probably due to electrical isolation of the microelectrode from integrating regions of the neuron by soma penetration (Wine et al. 1974).

No dye-coupling between the motoneurons was found following the Lucifer injection. No electrotonically spread spikes from other motoneurons were detected with a signal averaged intracellular recording from the motoneurons. The results suggest the possibility that the flexor motoneurons interacted through a chemically mediated synaptic connection, but not by electrical coupling through a gap junction. However, I could not totally eliminate the possibility of the existence of electrical synapses among the motoneurons because electrical events occurring far from the recording site will be drastically attenuated by non-electrogenic processes. Further study, for example manipulation of the Ca^{2+}/Mg^{2+} ionic level in the

ganglion, would be necessary to differentiate between electrical and chemical connections.

Wine et al. (1974) suggested that contralaterally homologous motoneurons shared their dendritic domains and had close apposition of their dorsal processes and neurites. They also postulated that the close apposition was a morphological basis of the coupling between motoneurons. In this study, I could show the expected close apposition of neuronal processes by using a differential staining technique. Although the close apposition does not directly indicate synaptic contact, the present result helps us to interpret an electrophysiologically observed connectivity.

I could show a possibility that the motoneurons are directly connected via their dorsal processes and neurites by differential staining, though a definitive demonstration of a monosynaptic connection must be provided by use of electron microscopy. However, localizing the site of close contact between neurons at the light microscopic level may be very helpful for further electron microscopic studies (Watson and Burrows 1982). In this investigation, contralaterally homologous motoneurons have extensive points of close contact on their dorsal processes located in dorsal commissure 3 (DC3) and on their neurites in the dorsal intermediate tracts (DIT). Further electron microscopic study should be concentrated on these regions to reveal the existence of monosynaptic connections between the motoneurons.

This intracellular study showed that several slow flexor motoneurons exert a premotor effect on their synergistic motoneurons confirming the conclusions of the spike train analysis (Sokolove and Tatton 1975; Tatton and Sokolove 1975). A motoneuron, once activated or inhibited, can recruit its synergists to perform in a well-coordinated contraction through these mutual couplings in the production and maintenance of abdominal posture movements. Slow flexor motoneurons of crayfish not only activate muscles distant from the ganglion, but also can function as integrative elements within the ganglion. Further study to characterize the

properties of other abdominal postural motoneurons, such as F3, may be necessary to understand their modulatory function in the neuronal circuit for abdominal postural movements in crayfish.

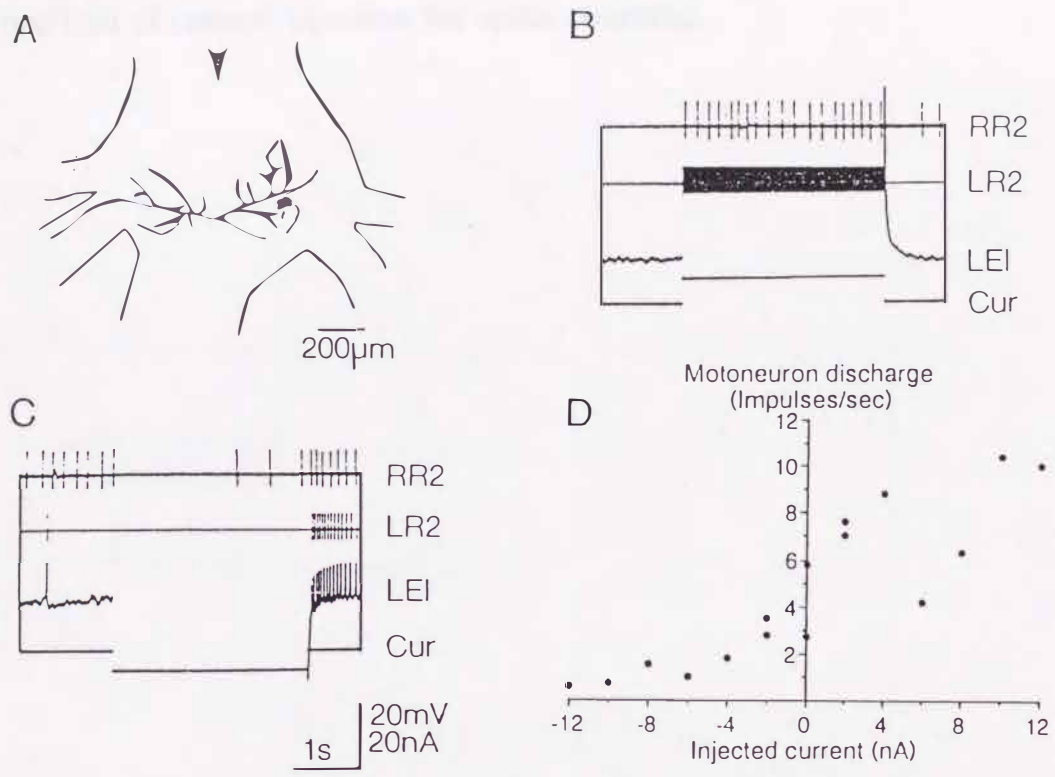

Figure 1. Connection between slow extensor inhibitory motoneurons. A: morphology of the slow extensor inhibitory motoneuron (EI) and its location in the ganglion. Anterior is at the top. Arrowhead indicates the midline. Ventral view. B: injection of a depolarizing current into the EI activated its own spike discharge (second trace) and that of the contralaterally homologous EI (top trace) in a synergistic way. C: injection of a hyperpolarizing current inhibited spike discharge of both EIs. RR2, motor activity in the right second root; LR2, the left second root; LEI, intracellular recording from the left EI; Cur, monitor of intracellular current injection. Upward deflection of the monitor indicates injection of a depolarizing current, and vice versa. Bridges are not balanced. D: relation between the intensity of injected current into EI and the spike frequency of the contralateral EI. Output effect on the contralateral EI depended on the polarity of the injected current and appeared to be either excitatory or inhibitory. In this

and the following graphs for current-spike correlation, each dot indicates the result from one trial of current injection for spike counting.

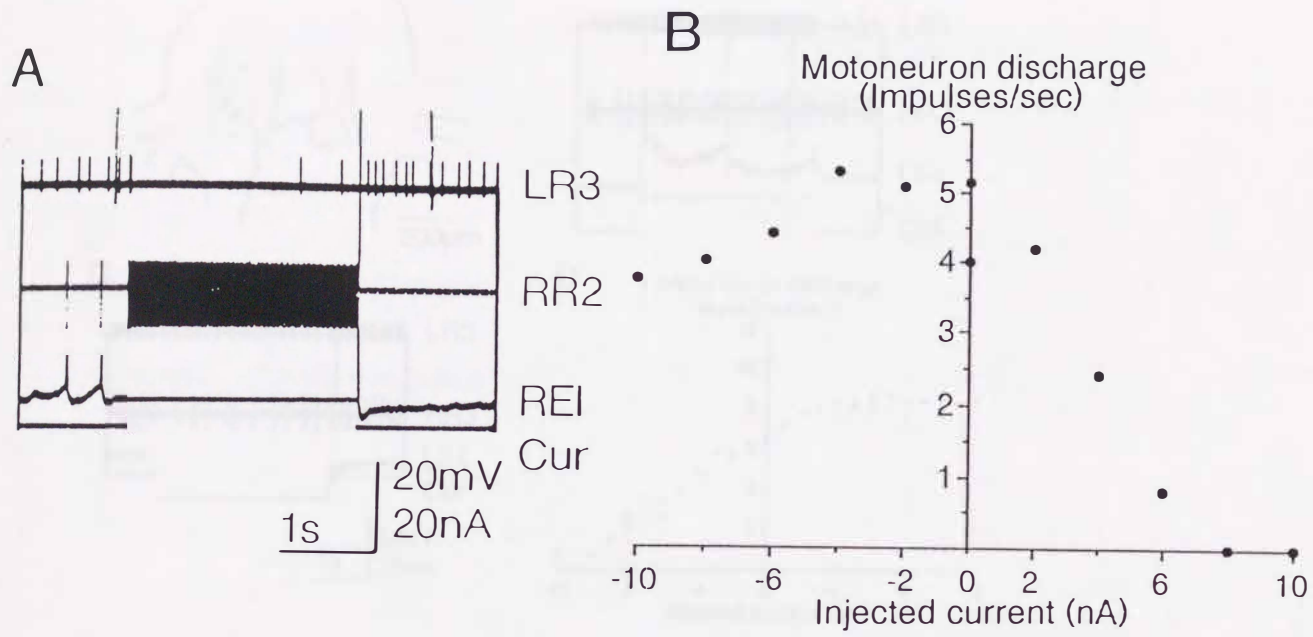

Figure 2. Connection among EI and the flexor motoneurons. A: injection of a depolarizing current into EI depressed the spontaneous spike discharge of the slow flexor excitatory motoneurons in the contralateral superficial third root (top trace). LR3, motor activity in the left superficial third root; REI, intracellular recording from the right EI. B: relation between the intensity of injected current into the REI and the spike frequency of LR3. Injection of a hyperpolarizing current had no noticeable effect on the flexor units.

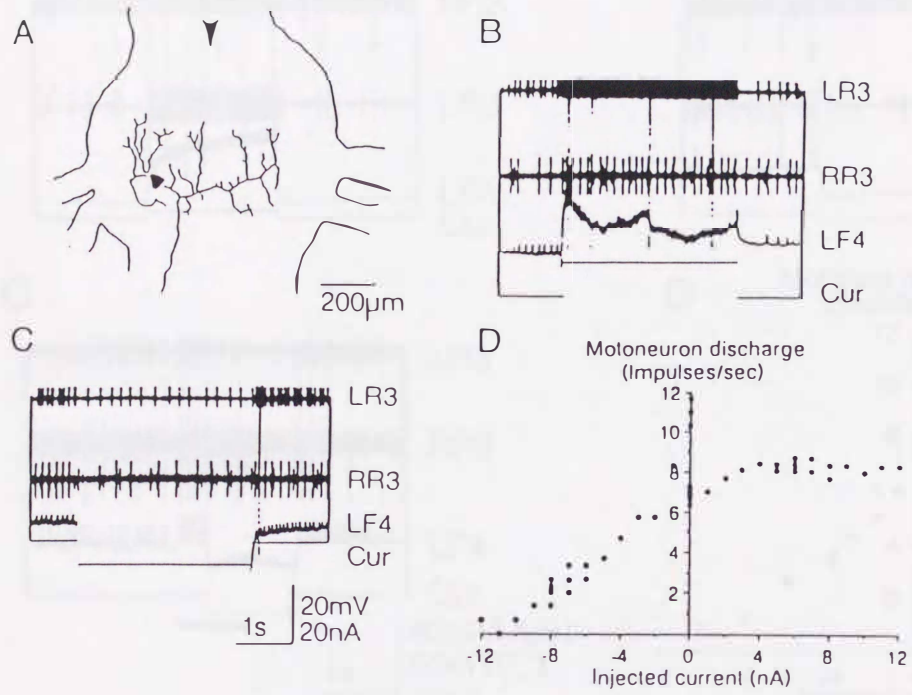

Figure 3. Connection between slow flexor No. 4. A: morphology of F4 and its location in the ganglion. The cell has bilateral neurites and postero-lateral soma in the ventral rind of the ganglion. Anterior is at the top. Arrowhead indicates the midline. Ventral view. B: injection of a depolarizing current into F4 activated spike discharge of contralateral F3 and/or F4 of middle-sized spike amplitude (second trace). The largest spike unit on the second trace is F6. C: injection of a hyperpolarizing current depressed spike discharge of the middle sized units (second trace). LF4, intracellular recording from the left flexor motoneuron No. 4. D: relation between the intensity of injected current into the F4 and the spike frequency of the contralateral units of middle-sized amplitude. Contralateral F3 and F4 were counted together in this figure because of their indistinguishable spike amplitudes.

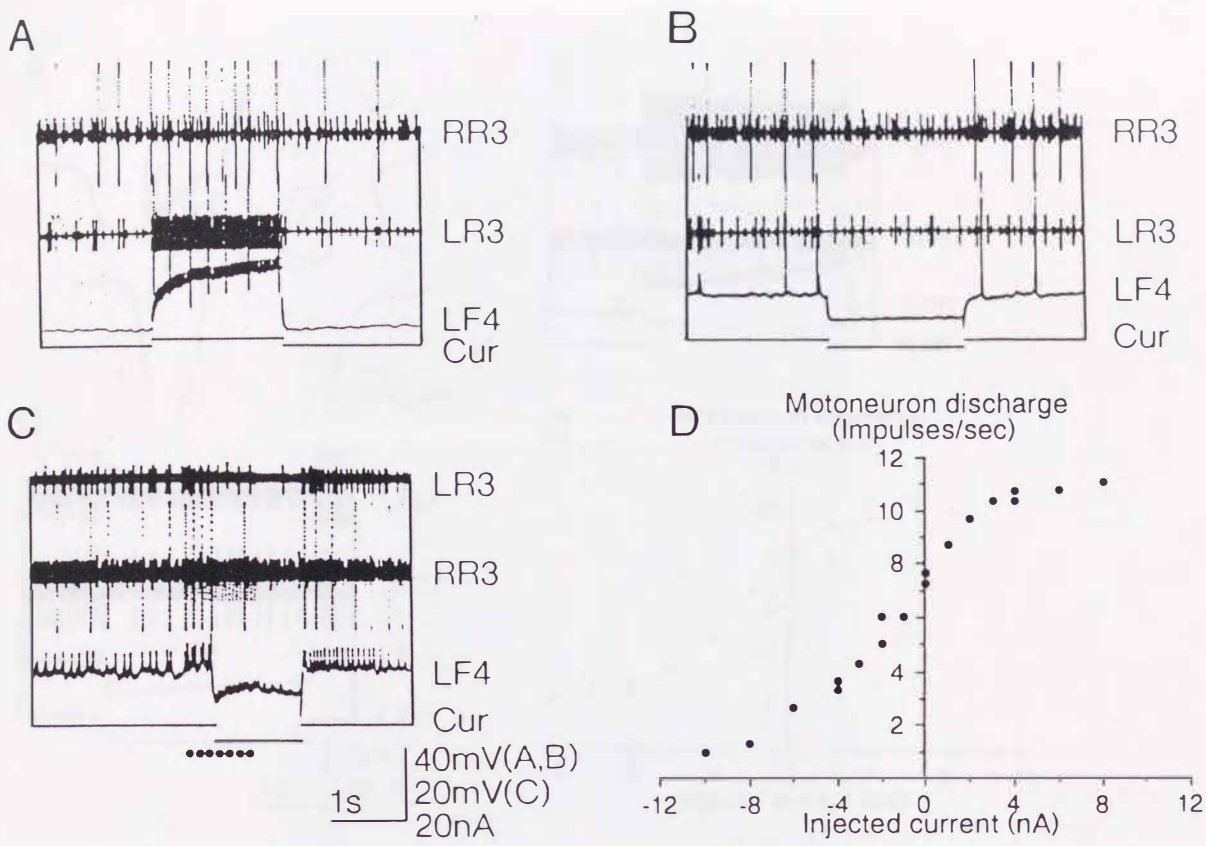

Figure 4. Connection among F4 and F6s in both sides. A, B: intracellular current injection into the F4 caused activation (A) or inhibition (B) of spike discharges of the F6s in a synergistic way. The largest units in extracellular recordings are F6s. C: F6 was artificially activated by electrical stimulation (6V, 0.1m sec pulse duration at 20 Hz) of the left fourth root in the terminal ganglion (second trace). Injection of a hyperpolarizing current into the contralateral F4 canceled both the spontaneous firing of the F4 itself (upper and third traces) and of the contralateral F6 (second trace). Dots indicate the duration of extracellularly applied electrical stimulation for F6 activation. D: relation between the intensity of injected current into the F4 and the spike frequency of the ipsilateral F6.

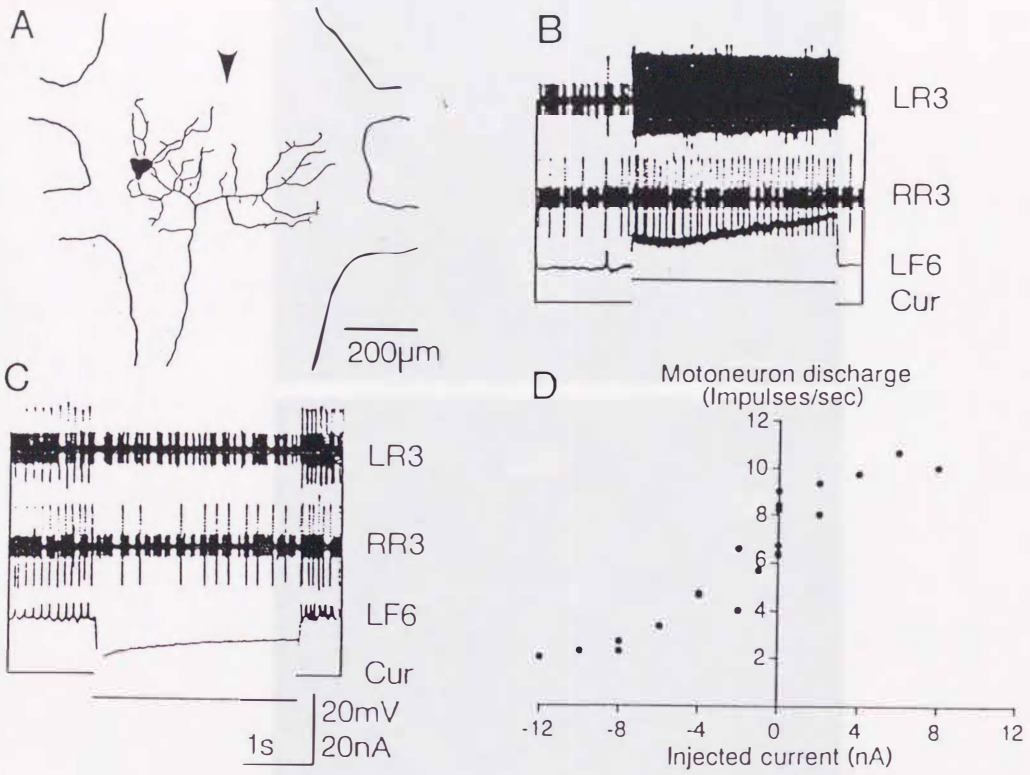

Figure 5. Connection between F6s in both sides. A: morphology and location of F6 in the ganglion. The cell had bilateral neurites and postero-lateral soma on the ventral rind of the ganglion. Anterior is at the top. Arrowhead indicates the midline. Ventral view. B: injection of a depolarizing current into the F6 activated its own spike discharge (largest unit on the top trace) and that of the contralateral F6 (largest unit on the second trace). LF6, intracellular recording from the left flexor motoneuron No.6. C: injection of a hyperpolarizing current depressed spike discharge of F6s in both sides. The current injection also depressed the spike discharge rate of F3 and/or F4 of middle-sized amplitude in both sides. D: relation between the intensity of injected current into the F6 and the spike frequency of the contralateral F6.

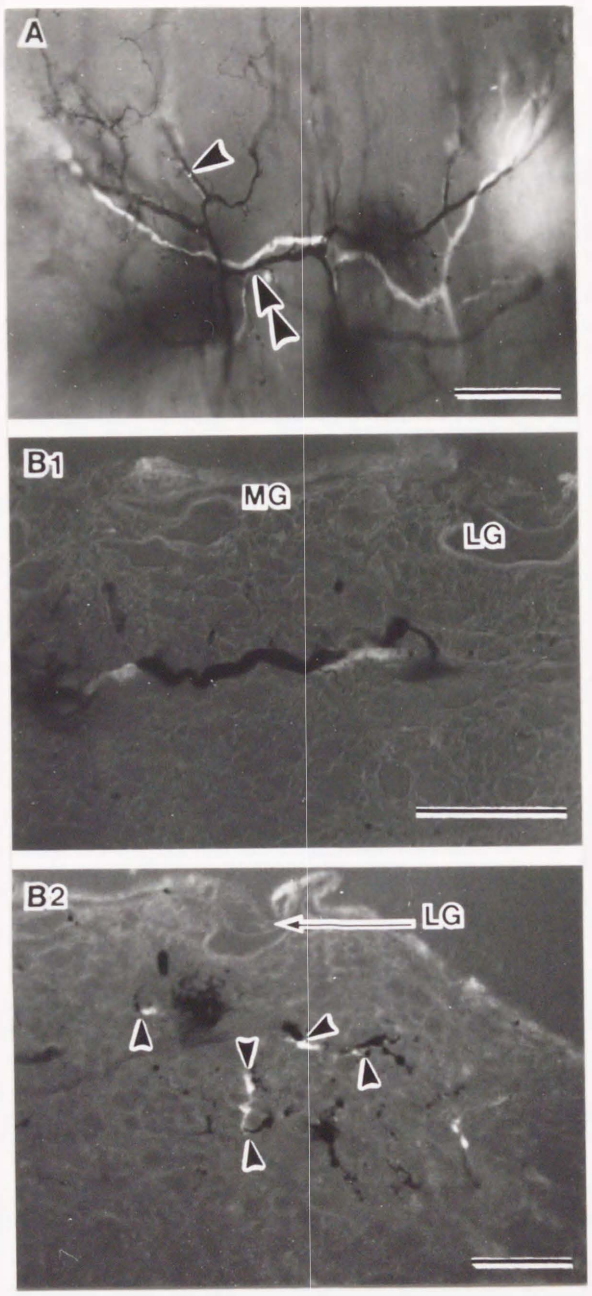

Figure 6. Presumed synaptic contacts between F4s under fluorescence microscopy. F4s in both sides were differentially stained with horseradish peroxidase and Lucifer yellow. Some processes of differently colored images are in close proximity of presumed synaptic site. A: whole mount preparation. Processes indicated by an arrowhead lie adjacent each other in this focal plane. Double arrowheads indicate possible contact between thick dorsal processes across the midline. Dorsal view. Anterior is at the top. B: transverse sections of the

ganglion shown in A. B1: proximity of the thick dorsal processes of the homologues. Differentially marked dorsal processes are located closely to one another in the same region in the ganglion. B2: possible contacts between neurites in another section. Arrowheads indicate the sites of possible contacts. MG: medial giant axon. LG: lateral giant axon. Scale bar: A 100 μm; B 50 μm

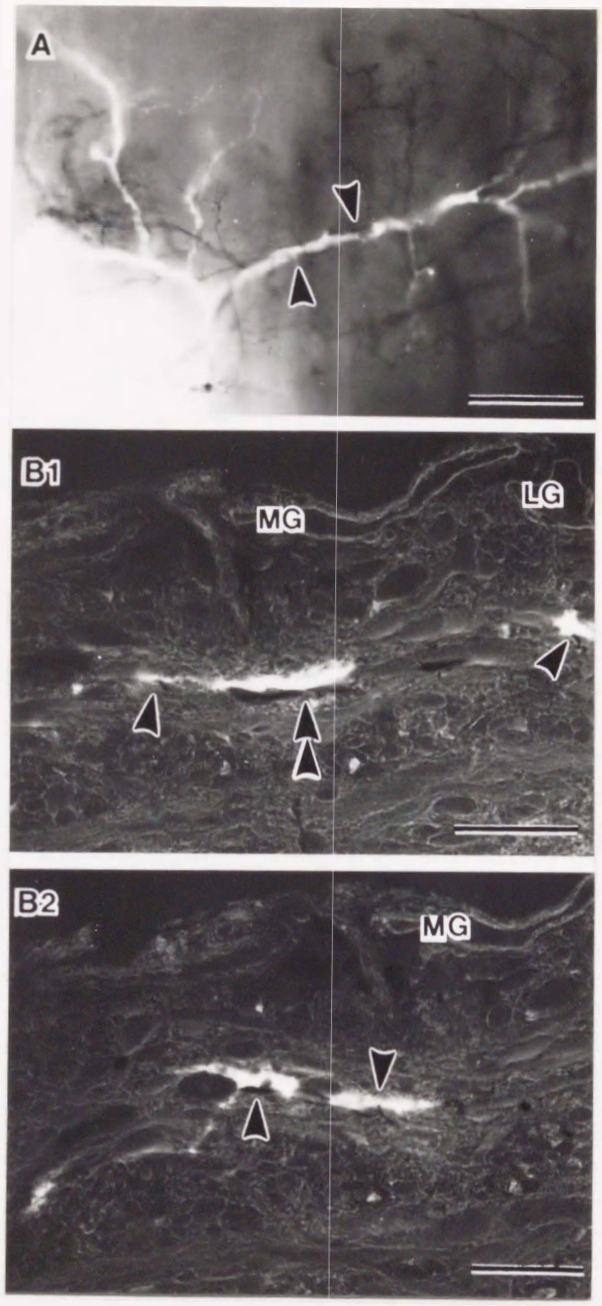

Figure 7. Presumed synaptic contacts between F6s. A: whole mount preparation. A thin process of darker image stained with horseradish peroxidase overlies the thick dorsal process stained with Lucifer yellow (indicated by arrowheads). Dorsal view. Anterior is at the top. B: transverse sections of the ganglion shown in A. B1: proximity of the thick dorsal processes of the homologues (double arrowheads). Single arrowheads indicate other possible contacts between neurites. B2: possible contacts between the thin process of the darker image and thick

dorsal process of the lighter one indicated by arrowheads on A. Scale bar: A 100 μm; B 50 μm

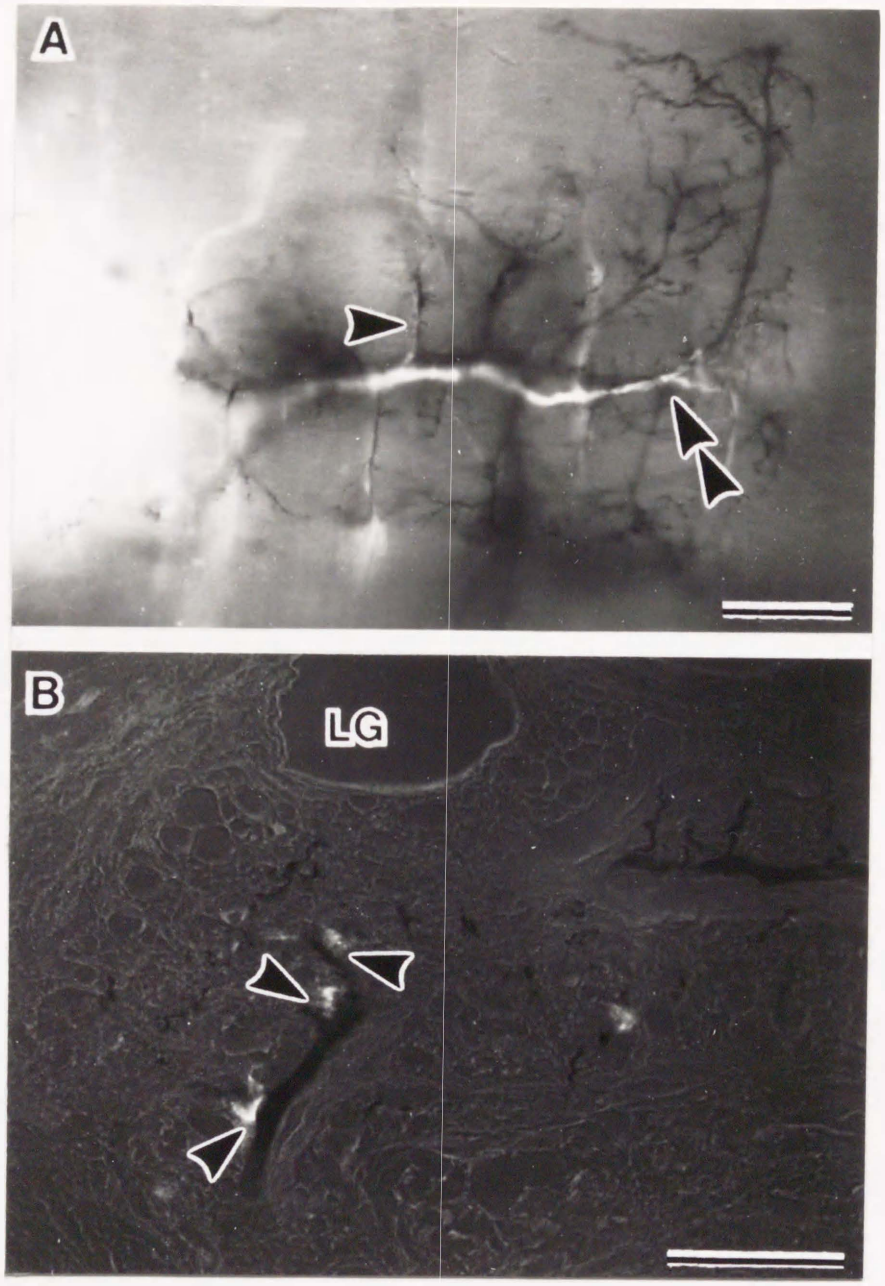

Figure 8. Presumed contacts between F4 and F6. A: whole mount preparation. The darker processes stained with horseradish peroxidase belong to the F6, while the lighter stained with Lucifer yellow are the contralateral F4. Two thin processes indicated by an arrowhead lie adjacent each other at the presumed synaptic site. Double arrowheads indicate possible contact between thick dorsal processes in this focal plane. Dorsal view. Anterior is at the top. B: a section from the ganglion shown in A. This section contains the processes indicated by

double arrowheads on A. Arrowheads indicate the possible contacts between the processes. Scale bar: A, 100 μm; B, 50 μm

Table 1. Summarizing results combined with those of extracellular analysis by Tatton and Sokolove (1975).

		Excitatory coupling		Inhibitory coupling	
driver	driven	T&S(1975)	This study	T&S(1975)	This study
EI	cEI	yes	yes	–	yes
F4	cF4	–	yes	–	yes
	iF6	–	yes	–	yes
	cF6	–	yes	–	yes
F6	iF4	yes	?	–	yes
	cF4	yes	yes	–	yes
	cF6	yes	yes	yes	yes

–: results not shown in Tatton and Sokolove (1975); ?: not examined; c:contralateral; i: ipsilateral, for example, cF4 indicates contralateral F4 cell.

General Discussion

This thesis revealed that local nonspiking interneurons were involved in the control of extension movement of crayfish abdomen. Nonspiking interneurons have been reported not only in the rhythmic motor systems but also in the motor control systems for episodic behavior. In episodic behavior, the interneurons function in the positioning of hindlegs in locust (Burrows 1980; Siegler 1985), compensatory eye movement (Okada and Yamaguchi 1988) and uropod steering movement (Nagayama et al. 1984) in crayfish. In these behaviors, the precise and continuous regulation of motoneuron discharge over a wide range will be necessary for mediation or modulation of behavioral acts. Burrows (1983) pointed out a possibility that the regulation was effectively achieved by nonspiking transmission. The same possibility could be applied to other episodic behavior of abdominal positioning in crayfish. The present finding that local nonspiking interneurons, LB cells and other unilateral cells, are involved in the control of abdominal positioning supports this possibility.

The crayfish uropod steering response to body rolling, one of the episodic behavioral acts, is facilitated effectively by interaction with abdominal posture movements (Takahata et al. 1981b). Local nonspiking interneurons in the terminal abdominal ganglion receive spike signals from the descending abdominal extension interneurons and in turn evoke subthreshold depolarization in the uropod motoneurons (Takahata and Hisada 1986 a, b). The newly identified LB cells also receive synaptic input from the abdominal extension interneurons (Figs. 5 and 6 in chapter I). LB cells, however, do not drive motoneuron activities by their artificial membrane potential change. Possible role of LB cells is quite different from that of local nonspiking interneurons which are functioning in gating of the descending motor pathway in the terminal abdominal ganglion (Takahata and Hisada 1986b). According to the notion of mediation-modulation dichotomy (Krasne et al. 1979), LB cells should be categorized under "modulation."

As noted above, LB cells do not directly mediate extension movement. Real extension movement may be mediated by interganglionic interneurons or by other unknown nonspiking interneurons (Fig.11 in chapter I). However, it is clear that LB cells contribute to the neuronal network for abdominal positioning, which has been hypothesized previously (Larimer 1988; Murphy et al. 1989).

The laser light epiillumination technique is used to ablate the entire or a partial region of a Lucifer-filled neuron. By using this technique, *in situ* axotomy of EI was accomplished during electrode penetration. The axotomized cell still maintained its output connection with its contralateral homologue (Fig.4 in chapter II). This indicates that dendrites of postural motoneurons function as premotor elements in the ganglion. There is a possibility that postural motoneurons operate not only as an output pathway for neural signals from the ganglion to muscles but also as a part of neural integration circuit in motor control systems for abdominal positioning.

Couplings among abdominal postural motoneurons have been indicated by several investigators (Evoy et al. 1967; Tatton and Sokolove 1975). These couplings were investigated using the antidromic stimulation technique and extracellular spike train analysis. It is the first time that in this thesis intracellular recording and staining technique were used to analyze these couplings among the motoneurons. By using the new technique which includes differential staining of neurons, these couplings could be observed physiologically and morphologically. A motoneuron exerted a premotor effect on its synergists through its dendrites, and in most cases the effect was bi-directional (Figs 4-8 in chapter III).

The inhibitory effect of a motoneuron induced by the injection of a hyperpolarizing current does not require its own spike generation. Couplings among motoneurons appear to be mediated by a chemical synaptic transmission (see chapter III). There is a possibility that dendrites of the postural motoneurons function as local processing elements such as the local nonspiking interneurons. In

uropod motoneurons, this possibility has already been suggested (Nagayama et al. 1984).

Although the crayfish abdominal positioning system has been generally understood in terms of "command fibers," I showed that the integral system consists of three different class of neurons: interganglionic interneurons (so called command fibers), intraganglionic interneurons and postural motoneurons. Further study to describe the connections among these different classes of neurons is necessary to understand the neuronal mechanisms underlying the abdominal positioning behavior in crayfish.

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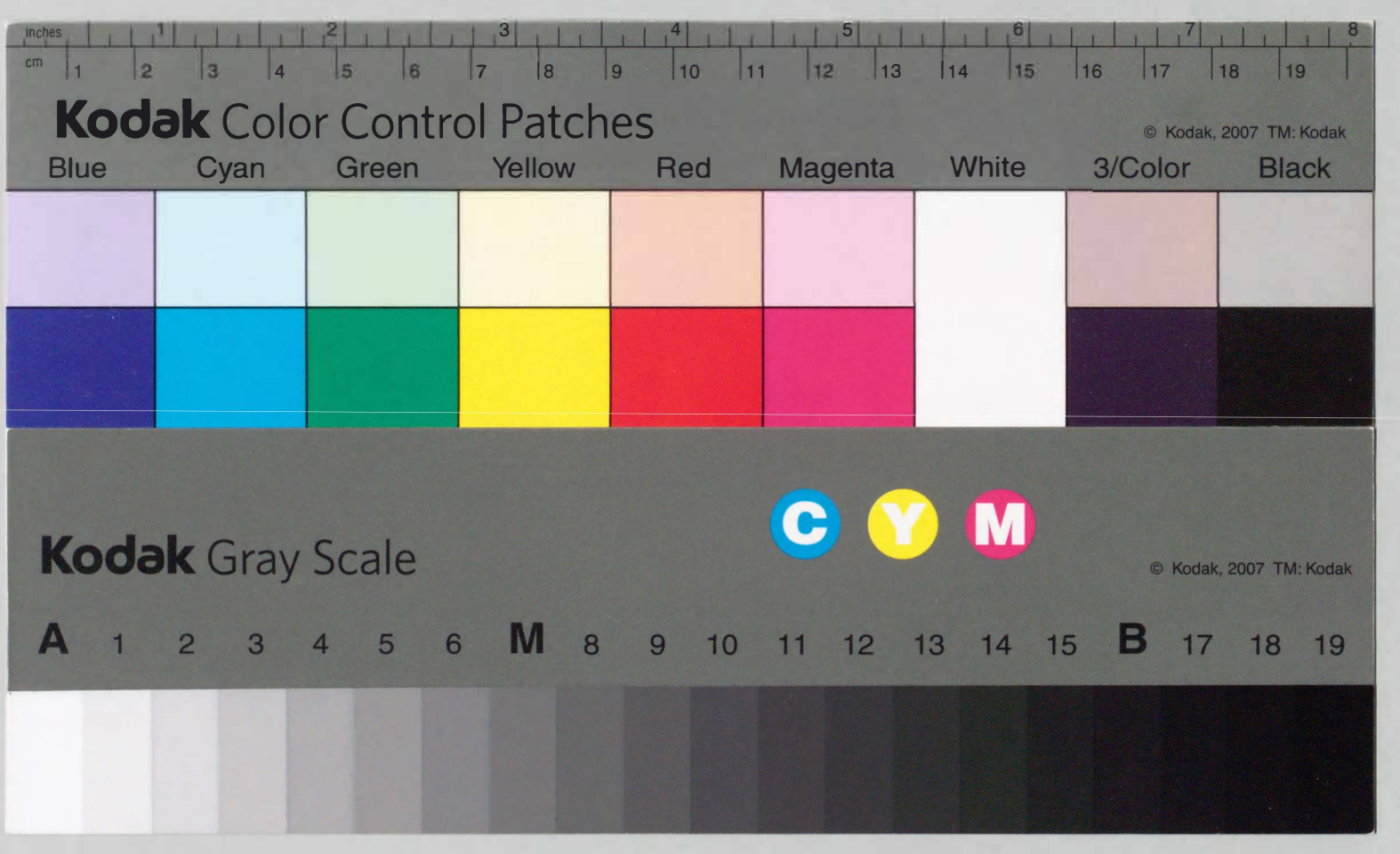
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