J. comp. Physiol. 105, 83—102 (1976)

Journal of Comparative Physiology · A

Crayfish Mechanoreceptive Interneurons:

I. The Nature of Ipsilateral Excitatory Inputs

Ronald L. Calabrese*

Department of Biological Sciences, Stanford University, Stanford, California, USA

Received August 4, 1975

Summary. 1. Cobalt impregnations of the crayfish, *Procambarus*, mechanoreceptive afferents and interneurons (Fig. 2) indicate that the afferents end with little terminal branching within the ipsilateral ganglionic neuropile in the area of the dendritic arbors of the interneurons.

2. The mechanoreceptive interneurons can be divided into two distinct classes: those that receive excitatory input only via direct afferent connections (primary interneurons) and those that, while they do receive direct afferent connections, receive the majority of their excitatory input from other interneurons (higher-order interneurons) (Figs. 4, 7–9, Table 2).

3. The connections between afferents and primary interneurons are probabilistic in nature; not all of the tactile afferents from the receptive field of a particular interneuron make functional synaptic contacts with that interneuron, nor do all interneurons that share a receptor surface in their receptive fields receive functional synaptic contacts from the same afferents arising from that surface (Fig. 6, Table 1).

4. Interneuronal input onto higher order interneurons amplifies the impulse bursts arising from phasic mechanical stimuli. Excitation is spread among a pool of interconnected interneurons, leading to prolonged and synchronous bursting following temporally restricted afferent volleys (Figs. 7, 8).

Introduction

Crayfish mechanoreceptive[1] hair afferents and the large interneurons of the abdominal nerve cord which they innervate, comprise the afferent wing of the animal's dramatic backward escape reflex. This reflex is initiated by phasic mechanical stimuli to the abdomen and is mediated via the lateral giant fibers (Wiersma, 1947; Wiersma and Hughes, 1961; Krasne, 1969; Zucker, Kennedy and Selverston, 1971; Zucker, 1972; Wine and Krasne, 1972). Because of their importance to the behavior of the animal and the ease with which they are recorded these neurons have attracted much attention from neurophysiologists.

Pioneering studies by Wiersma and co-workers (Hughes and Wiersma, 1960; Wiersma and Hughes, 1961) established the uniqueness and identifiability of these interneurons by demonstrating constancy in their receptive fields and their position in the interganglionic connectives. Several workers (Preston and Kennedy, 1960; Kennedy and Mellon, 1964; Zucker, 1972; Wilkens and Larimer, 1972) have shown that these cells are easily penetrated within the ganglionic neuropile with micropipettes. Such recording techniques have permitted the physiological analysis of mechanisms responsible for the synaptic integration of mechanoreceptive input (Kennedy and Mellon, 1964; Zucker, 1972), and the advent of intracellular dye injection techniques (Stretton and Kravitz, 1968; Pitman,

* Present address: Department of Molecular Biology, University of California, Berkeley, California 94720, USA.

1 These hair afferents are best termed mechanoreceptive as they respond to near field water disturbances (Wiese, in preparation) as well as to direct touch.

Tweedle and Cohen, 1972) has made possible a detailed anatomical reconstruction of several of these interneurons (Selverston and Kennedy, 1969; Kennedy, 1971; Wilkens and Larimer, 1972). Similarly the mechanoreceptive hairs on the proximal telson have been mapped individually so that they can usually be recognized from animal to animal (Kennedy, 1971, 1974), and the identifiability of these afferents has been used to determine the consistancy of their connectivity with an identified interneuron-interneuron A (Zucker, Kennedy and Selverston, 1971; Kennedy, 1971, 1974).

A number of issues regarding the connectivity and synaptic interactions of this neuron population are nevertheless still unresolved. The central anatomy of the hair afferents is not known, and the functional significance of their central projections in the interganglionic connectives (Hughes and Wiersma, 1960; Wiersma and Hughes, 1961) is unclear. All mechanoreceptive interneurons which receive sensory input from more than one abdominal segment receive afferent connections and generate spikes in each ganglion to which the hair afferents of their receptive fields project (Hughes and Wiersma, 1960; Kennedy and Mellon, 1964). Nevertheless, the central projection of tactile afferents in the interganglionic connectives may be involved in intersegmental summation (Kennedy and Mellon, 1964).

The connectivity between telson hair afferents and interneuron A mentioned above has been shown not to be completely consistent from preparation to preparation, as are many of the central connections between interneurons and motor neurons in the same system (Kennedy, 1971, 1974). Identified telson hair afferents do not always connect with interneuron A. Hair afferents from the rostral telson are more likely to connect with interneuron A than those from the caudal telson in any given preparation, but few afferents connect with interneuron A in every preparation (Kennedy, 1971, 1974). Thus it appears that a given hair afferent can only be assigned some probability of connecting with interneuron A in any given preparation. It is not known, however, whether this "hit and miss" pattern is a general property of the connectivity between mechanoreceptive afferents and all their interneurons, or whether the failure of an afferent to make connection with a particular interneuron is an indication that it will fail to make connection with other interneurons that are driven from the same receptive field. Nor is it known whether afferents which project into the interganglionic connectives represent neurons that have failed to connect with interneurons within their ganglion of entry.

Finally the hierarchical organization of mechanoreceptive interneurons remains unclear. Zucker (1972, 1973) has shown that interneurons with broad receptive fields receive excitatory input from those with more restricted receptive fields. However, the extent and functional significance of this interneuronal interaction has not been fully evaluated.

These issues are addressed in this paper. In particular, the experiments pertain to the ipsilateral connectivity patterns of telson mechanoreceptive afferents and interneurons in the sixth abdominal ganglion of the crayfish *Procambarus clarkii*.

Methods

Procambarus clarkii were used for all experiments. Male and female specimens (5–10 cm in length) were obtained from local suppliers or collected from streams, and maintained in aerated tap water until used.

Anatomy

Interneurons, motor neurons, and sensory neurons in isolated abdominal nerve cords were filled with cobaltous chloride (175 mM—hyposmotic—or 300 mM—hyperosmotic—in distilled water) by diffusion into the cut end of an interganglionic connective or ganglionic root or by sealing the cut end in a polyvinylchloride (PVC) suction electrode filled with cobalt solution and passing a positive DC current of ten microamps. After 12–16 hours at approximately 8° C, the intracellular cobalt was precipitated with ammonium sulphide (Pitman, Tweedle and Cohen, 1972). Occasionally longer injection times—up to 48 hr—were used with no significant differences in results. The abdominal nerve cord was then fixed in 10% formalin in van Harreveld's solution (van Harreveld, 1936), dehydrated in a series of alcohols, and cleared in methyl salicylate. Whole mounts in methyl salicylate were photographed through a Wild dissecting photomicroscope.

Soft cuticular pressure receptors (Pabst and Kennedy, 1967) were stained in formol-saline fixed abdominal nerve cords with Azure B, dehydrated, and observed in cleared whole mounts with a standard compound microscope.

Dissections

Before dissection the animals were immobilized by cooling in ice for approximately 10–15 min. Early extracellular experiments were performed on the split abdomen preparation described by Zucker (1972). All intracellular experiments employed a semi-isolated abdominal nerve cord preparation that included the uropods and telson. The abdominal nerve cord was isolated from the animal except for its connections to the telson via one or both of the pair of roots of the sixth ganglion which innervate it. Unless otherwise stated in the text, only the fourth roots were left intact. The sixth segment appendages were pinned out dorsal side up (allowing mechanical access to the mechanoreceptive hairs on the dorsal surface of the telson) in a plastic chamber containing a clear Sylgard bottom in which a mirror had been embedded to allow illumination from below. The chamber was filled with cold van Harreveld's solution (van Harreveld, 1936) with the bicarbonate buffer replaced by Trizma and adjusted to pH 7.2. The roots of the sixth ganglion were twisted, the nerve cord pinned out ventral surface up, and the connective tissue sheath removed from the 5/6 interganglionic connectives and the ventral surface of the sixth ganglion. Individual axons or small bundles of axons were then sometimes dissected free from the desheathed interganglionic connectives for subsequent recording and/or stimulation and identification.

Recordings

Suction electrodes were used for all extracellular recordings and for electrical stimulation. They were led to a switch box that allowed any electrode to be used for either stimulation or recording. Extracellular recordings were amplified with capacity-coupled preamplifiers, and electrical stimuli were provided by pulse generating equipment with outputs isolated from ground by transformers.

Intracellular recordings were made with micropipettes pulled with glass fibers and filled with 3 M KCl. Such electrodes had resistances of between 30 and 80 MΩ measured in the physiological saline. Signals were recorded using a WPI capacitance-compensated high impedance DC preamplifier, with a "bootstrap" bridge for passing current through the recording microelectrode. A Grass SD9 stimulator was used in conjunction with the bridge.

Using known ganglionic landmarks as a guide, the neuropile of the sixth ganglion was probed with a microelectrode until units with the desired characteristics were encountered. The hunt for interneuronal dendritic processes was facilitated by cobalt impregnations of the 5/6 interganglionic connective, in which the interneuronal axons run, revealing the neuropilar areas in which the interneuronal processes are concentrated (Fig. 2A). Penetrated units were discarded unless they had stable resting potentials of 40 mV or greater.

Quasi-intracellular recordings were made from interneuron A (Zucker, Kennedy and Selverston, 1971) with a small suction electrode into which the isolated axon of the interneuron was drawn just to its point of emergence from the sixth ganglion (Kennedy, 1971). Under such conditions, stable unitary EPSP's, compound IPSP's and spike activity could be recorded for periods of ten minutes to one hour.

Identification of Units

A unit penetrated within the ganglionic neuropile was judged to be an interneuron if it could be shown to have an axon in the interganglionic connectives and responded readily with EPSP's and/or spikes to tactile stimulation of a large area of the telson and electrical stimulation of an afferent root. (Latencies of EPSP's in interneurons were calculated from the arrival time of afferent spikes in the 6th ganglion neuropile (cf. Zucker, 1972)). Certain interneuronal elements penetrated within the ganglion were readily identifiable by their receptive field properties, the position and size of their axons in the interganglionic connectives, and their characteristic discharge pattern in response to electrical stimulation of an afferent root. Two such identifiable units were regularly encountered; interneuron A and interneuron C. Interneuron A is the largest fiber in the abdominal nerve cord other than the giant fibers; it has a receptive field which includes the ipsilateral uropods and telson (Zucker, Kennedy and Selverston, 1971), and is equivalent to fiber A6 of Wiersma and Hughes (1961). Interneuron C is another very large and prominent fiber in the abdominal nerve cord; it has a receptive field which includes the entire dorsal surface of the abdomen (Zucker, Kennedy and Selverston, 1971) and is equivalent to fiber A64 of Wiersma and Hughes (1961). Similar criteria were applied to the identification of interneurons in extracellular records from the interganglionic connectives.

Fourth and sometimes fifth root mechanoreceptive afferent fibers were catalogued in a particular preparation by their extracellularly recorded spike amplitude and waveform and by their characteristic spontaneous discharge frequency. Individual fourth root afferents could be stimulated by moving single identified hairs on the telson (Kennedy, 1971). Neurons penetrated in the sixth ganglion neuropile were judged to be fourth root mechanoreceptive afferents if they met the criteria set out by Kennedy *et al.* (1974).

Results

The Receptive Fields of Sixth Ganglion Sensory Roots

A large number of mechanoreceptive hairs occupy the two surfaces of the sixth abdominal segment and its appendages—the uropods and telson—and send their axons into the central nervous system via the roots of the sixth ganglion. The receptive fields of the various roots have been mapped, as shown in Fig. 1, by recording responses to mechanical stimulation with a fine brush. They are strictly ipsilateral and non-overlapping.

The Composition of the Fourth Root and the Anatomy of Its Mechanoreceptive Afferents

The fourth root contains a number of large axons (5–10 microns in diameter) (Kennedy, 1974), many of which are associated with the mechanoreceptive hairs on the dorsal surface of the laterorostral telson. These axons enter the main root via its medial branch. Several axons of large diameter form a smaller lateral branch; these are associated with mechanoreceptive hairs on the caudal edge of the dorsal surface of the sixth abdominal segment (Fig. 1). The root also contains the cell bodies of several neurons that are morphologically identical to the soft cuticular pressure receptors described by Pabst and Kennedy (1967) in the roots of other abdominal ganglia.

Cobalt impregnations of this root reveal a single cell body in the ganglion which is medial and ipsilateral (Fig. 2B). In addition extracellular recordings from the fourth root reveal only one tonically active efferent unit in the root. Therefore, it seems safe to assume that the fourth root contains only a single efferent fiber. On only a few occasions, large neurons in addition to the single efferent fiber were filled with cobalt; these are assumed to be associated with

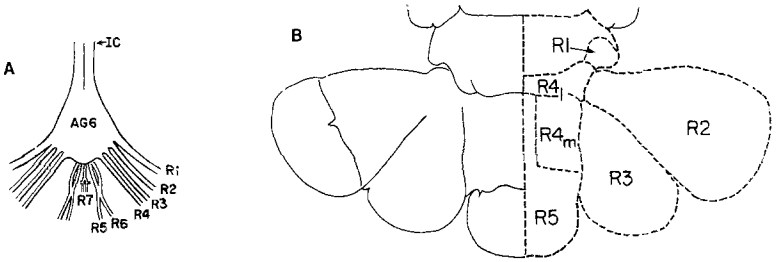

Fig. 1. (A) Composite diagram of the sixth abdominal ganglion of the crayfish, *Procambarus clarkii* (ventral aspect). The roots are numbered according to the scheme used by Larimer and Kennedy (1969). Root 7 is unpaired but all others have bilateral homologues. Both root 7 and root 6 lack mechanoreceptive afferent components. (B) Composite diagram of the sixth abdominal segment and its appendages—the uropods and telson—of the crayfish *Procambarus clarkii* (dorsal aspect), showing the mechanoreceptive fields of the various roots of the sixth abdominal ganglion. Receptive fields are strictly ipsilateral and non-overlapping. The lateral and medial branches of the fourth root ($R4_l$ and $R4_m$) are considered separately because they have quite different central projections. Ventral surface hair afferents, where they occur, enter the same root as their dorsal counterparts. Abbreviations used throughout: R = root. $A\,G\,6$ = sixth abdominal ganglion

the mechanoreceptive hair afferents because they have no cell bodies within the ganglion or the root (Fig. 2C and D). These fibers progress rostrally into the central neuropile into the vicinity of dense interneuronal dendritic arborizations (Fig. 2A), where they appear to end with little terminal branching (Fig. 2D). No cobalt-filled fourth root fiber has been observed to cross the ganglionic midline or enter the interganglionic connectives. Physiological evidence, presented in the next section shows that some mechanoreceptive hair afferents do in fact send axons into the interganglionic connectives; thus it may be that the afferent fibers do not fill completely with cobalt with the techniques used. The fifth root (for receptive field, see Fig. 1) appears not to contain any efferent fibers; otherwise, it resembles the fourth in composition[2] and in the central anatomy of its mechanoreceptive afferents. Cobalt fills of this root have a similar low probability of success.

Central Projection of Fourth Root Mechanoreceptive Afferents

Electrical stimulation of the ventromedial aspect of the connective between the fifth and sixth ganglion leads to a powerful antidromic discharge in the ipsilateral but not the contralateral fourth root (Fig. 3A). The number of units responding is a function of the stimulus intensity; response components are added in an all-or-none fashion. The response has a short and constant latency and follows stimulus rates up to 100 Hz without failure (Fig. 3C). Electrical stimulation of the fourth root leads to a similar response from a fiber tract in

2 Barth (1964) has demonstrated the presence of fifth root afferents sensitive to telson displacement. These neurons, though present, are normally silent in the preparation used here. The fifth root fuses with the sixth just caudal to their exit from the ganglion, and thus some sixth root motor neurons occur in the fifth root peripheral to this fusion. In these experiments the sixth root was always severed proximal to its fusion with the fifth root (see Fig. 1A).

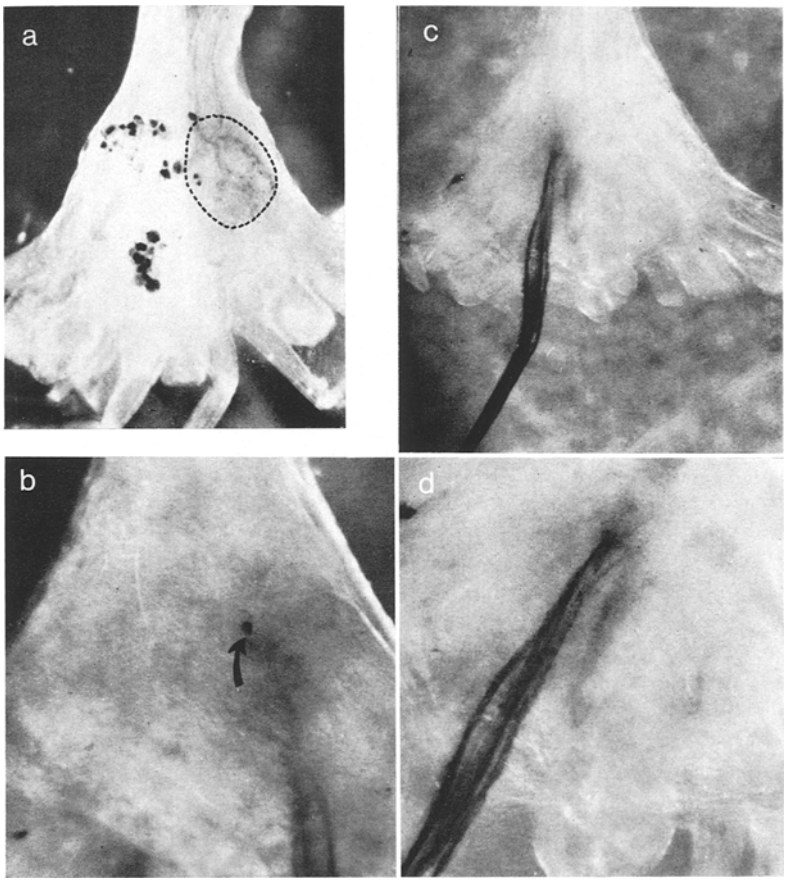

Fig. 2A—D. Anatomy of sixth ganglion mechanoreceptive afferents and interneurons as determined by cobalt dye impregnation. (A) Photograph of the ventral surface of the sixth ganglion in which the left interganglionic connective was filled with cobalt, revealing the cell bodies and dendritic ramifications of many mechanoreceptive interneurons. The dotted line indicates the ipsilateral dendritic domains of the interneurons. (B) Photograph of the ventral surface of the sixth ganglion in which the left fourth root has been filled with cobalt dye. The arrow indicates the cell body of the single efferent fiber regularly found in the fourth root. (C) Photograph of the dorsal surface of the same preparation as B at low power. The large axons which are filled with cobalt are presumably those of telson hair afferents. (D) Same as C except at higher power. Note that afferent fibers appear to terminate within the dendritic domain of the mechanoreceptive interneurons. Calibration bar: (A, C) 400 μM; (B, D) 200 μM

the ventromedial aspect of the adjacent ipsilateral but not the contralateral interganglionic connective (Fig. 3B and D). These observations have been confirmed by intracellular recording from fourth root mechanoreceptive afferents penetrated in the ganglionic neuropile (Fig. 3E and F). The antidromic response of a fourth root unit to connective stimulation may be blocked by orthodromic activity evoked in the same unit by deflecting the appropriate sensory hair. Afferents belonging to the fourth root thus must project to more rostral ganglia. Most of the afferents entering the interganglionic connectives are those of sixth

segment hairs that have axons of relatively large diameter (as judged by extracellularly recorded spike amplitude) in the lateral branch of the root (Fig. 3A); only a few of these axons project beyond the fifth ganglion. Afferents from telson hairs that enter the interganglionic connectives are of smaller diameter and originate in the caudal part of the fourth root telson field (Fig. 3A). The largest axons from the phasic hair afferents of the rostral fourth root telson field (Kennedy, 1971) cannot be shown to enter the interganglionic connective by the same techniques.

Antidromic spikes evoked in fourth root mechanoreceptive afferents by stimulating interganglionic connectives can give rise to unitary EPSP's in first-order mechanoreceptive interneurons within the sixth ganglion (Fig. 3G and H). In addition antidromic discharge in fourth root afferents evoked by interganglionic connective stimulation can produce interneuronal discharges which originate in the sixth ganglion—in particular, in interneuron A which is known to receive only direct mechanoreceptive afferent connections and only within the sixth ganglion (Zucker, Kennedy and Selverston, 1971). Thus afferent axons that project into the interganglionic connectives must make synapses within the sixth ganglion.

To compare the arrangement in more "typical" segmental ganglia, similar experiments were performed on second root mechanoreceptive hair afferents of the third abdominal ganglion. The axons of some of these sensory neurons may enter either the ascending or descending ipsilateral interganglionic connectives. The ascending projection is heavier than the descending one, in agreement with the observation that intersegmental summation effects onto multisegmental mechanoreceptive interneurons are rostrally biased (Kennedy and Mellon, 1964).

Connections between Mechanoreceptive Afferents and Primary Interneurons in the Sixth Ganglion

Neurons penetrated within the sixth ganglion were judged to be primary mechanoreceptive interneurons if they met the following criteria: (1) they responded with EPSP's to stimulation of a number of telson hairs. (2) they could be shown to have an axon in the interganglionic connectives. (3) unitary EPSP's followed the individual spikes of afferents in ipsilateral roots at a constant latency of less than 2 msec. (4) no unitary EPSP's in the cell were associated with interneuronal activity recorded in the interganglionic connectives. Of 25 mechanoreceptive interneurons sampled in four preparations in order to estimate the proportion of primary interneurons, only 5 met all criteria; one of these is illustrated in Fig. 4A. (The sample did not include interneuron A, discussed below.) Most primary interneurons encountered were tested for their response to electrical stimulation of the ipsilateral fourth root. They responded with short-latency (0.5–2 msec) compound EPSP's of relatively short duration (10–40 msec) and rarely produced more than 3 impulses even at maximal stimulus intensities (Fig. 4B). All primary interneurons tested also responded to fifth root stimulation. The short latency of compound EPSP's evoked by electrical stimulation of afferent roots and the observation that these EPSP's were increased in amplitude by increasing the level of polarization of the cell, indicates that they are monosynaptic and chemically mediated. These cells as a class are therefore

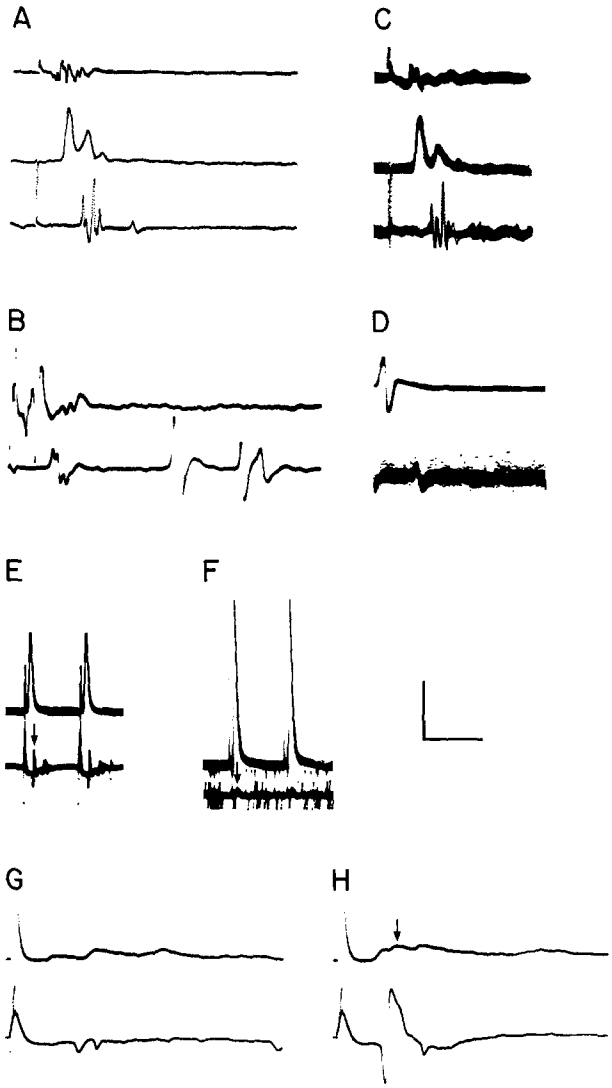

Fig. 3A—H. Central projections of fourth root mechanoreceptive afferents. (A, C) Antidromic spikes in fourth root afferents evoked by electrical stimulation of the ipsilateral interganglionic connective; a single stimulus and superimposed sweeps from a train of stimuli at 100 Hz, respectively. Traces are from top to bottom: main fourth root activity, activity in the lateral branch of the fourth root (spikes are monophasic because the recording is from the cut end of this nerve), activity in the medial branch of the fourth root. (B, D) Fourth root afferent activity recorded in the ipsilateral interganglionic connectives, evoked by electrical stimulation of the fourth root; a single stimulus and superimposed sweeps from a train of stimuli at 100 Hz, respectively. The top traces monitor fourth root activity, and the bottom traces monitor fourth root afferent activity in the interganglionic connective. In D the stimulus intensity was adjusted so that only a single unit responds in the interganglionic connectives. (E) Antidromic response evoked in a fourth root mechanoreceptive afferent fiber penetrated in the ganglionic neuropile by electrical stimulation of the ipsilateral interganglionic connective; superimposed sweeps from a strain of stimuli at 100 Hz. The top trace is an intracellular record from the mechanoreceptive afferent, and the bottom trace monitors activity in the same afferent in the fourth root (afferent spike indicated by arrow). (F) Activity recorded intracellularly from a fourth root mechanoreceptive afferent

similar to interneuron A (Kennedy, 1971; Zucker, Kennedy and Selverston, 1971), a primary interneuron also often encountered in the present experiments. Table 2 summarizes the response properties of primary interneurons.

Probabilistic Nature of Mechanoreceptive Afferent Connections onto Primary Interneurons

Kennedy (1971) has previously shown that fourth root afferents from telson hairs connect in a "probabilistic" fashion with interneuron A: that is, not all the afferents from within the receptive field of interneuron A connect with it, and the particular afferents that do so differ somewhat from preparation to preparation.

Since the judgement that a particular afferent connects or fails to connect with an interneuron requires the assumption that all the synaptic input onto a given interneuron can be recorded from a single site, experiments were performed to test this assumption. In these experiments, the identified axon of interneuron A was placed in a suction electrode so that unitary EPSP's associated with the discharge of single afferents from both fourth and fifth roots could be recorded. The neuropile of the ganglion was then probed with a microelectrode until a dendrite of interneuron A was penetrated: thus the two recording sites straddled the spike initiation zone.

Intracellular and extracellular records were identical in the two successful experiments of this kind, except that the extracellularly recorded EPSP's showed more capacitive distortion (Fig. 5A and B). The afferents that produced no EPSP's in one record also failed to show them in the other, and vice-versa. These results indicate that all of the synaptic inputs onto this tactile interneuron are within a space constant or so of one another and of the intracellular recording sites, perhaps because each afferent makes widely-distributed, multiple terminations. Since the unitary EPSP's recorded from various intracellular sites in other primary interneurons (Fig. 4A) are similar in amplitude and waveform to those recorded in interneuron A, all these cells probably have dendritic trees with roughly similar cable properties. Therefore, the assumption that it is possible to record all unitary EPSPs evoked in them by ipsilateral afferents seems quite reasonable.

Are afferents that fail to connect with a particular primary interneuron also ineffectual in making synaptic contacts with other primary interneurons that

penetrated in the ganglionic neuropile and extracellularly from the same fiber in the ipsilateral interganglionic connective (afferent spike indicated by arrow). The activity was evoked by electrical stimulation of the ipsilateral fourth root; superimposed sweeps from a train of stimuli at 100 Hz. The top trace is an intracellular record from the afferent and the bottom trace monitors afferent activity in the interganglionic connective. (G, H) Unitary EPSP (indicated by arrow in H), produced in a first-order interneuron by an antidromic spike in the fourth root mechanoreceptive afferent which was evoked by electrical stimulation of the ipsilateral interganglionic connective. In G the stimulus intensity is just below threshold for antidromic activity in the afferent; in H the stimulus intensity is just above threshold for antidromic activity in the afferent. The top trace is an intracellular record from the interneuron, and the bottom trace monitors fourth root activity. Calibration bars: (A, B, C) 2 msec; (D) 1 msec; (E) 10 mV, 5 msec; (F) 5 mV, 5 msec; (G, H) 2 mV, 2 msec. Voltage calibrations refer only to the intracellular traces

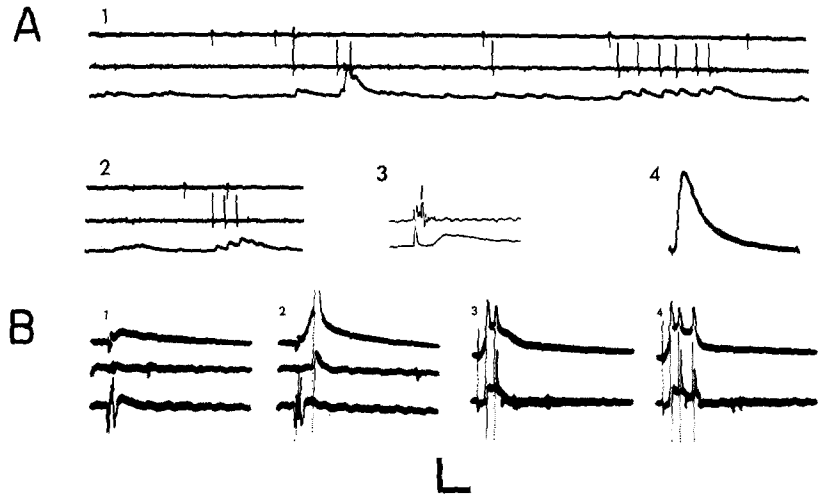

Fig. 4A and B. Records from an unidentified primary mechanoreceptive interneuron in the sixth ganglion. (A) 1) Mechanically evoked activity in the interneuron. Traces are from top to bottom: interneuronal activity recorded in the ipsilateral interganglionic connective, ipsilateral afferent root activity, and intracellular records from the interneuron. Note the two particularly active afferents (one is quite small), each of which gives rise to unitary EPSP's in the interneuron. 2) An excerpt from the same record as 1, demonstrating that high frequency activity in the small afferent produces a stable EPSP amplitude, whereas similar activity in the large afferent produces a facilitating EPSP. 3) Response of the interneuron to electrical stimulation of an ipsilateral afferent root. The top trace shows the afferent volley in the stimulated root, and the bottom trace is an intercellular record from the interneuron. 4) Superimposed sweeps of antidromic activity recorded intracellularly from the interneuron induced by electrical stimulation of the ipsilateral interganglionic connective at 100 Hz, demonstrating that the penetrated cell has an axon in the ipsilateral interganglionic connective. Interneurons appear to have no effect on the penetrated cell. (B) The response of a primary mechanoreceptive interneuron to electrical stimulation of an ipsilateral afferent root at four different increasing intensities (1 to 4, respectively). Traces are from top to bottom: Intracellular records from the interneuron, interneuronal activity recorded from the ipsilateral interganglionic connective, and ipsilateral afferent root activity. In 3 and 4 the bottom trace has been omitted. The large spikes in the connective record are from the axon of the interneuron. Calibration bars: (A) 1,2) 3.25 mV, 6.5 msec; 3) 3.25 mV, 1.3 msec; 4) 3.25 mV, 0.65 msec. (B) 1,2) 20 mV, 10 msec; 3,4) 40 mV, 20 msec. Voltage calibrations refer only to intracellular records. Afferent spikes in A were retouched

include the same receptor surface in their receptive fields? To answer this question, serial penetrations were made of primary interneurons in the same preparation while a constant population of afferents was sampled. The sensory units sampled were fourth root telson hair afferents showing activity in the absence of any applied stimulation. Since such discharge results from spontaneous surface waves in the preparation dish (Wiese, personal communication), the afferents sampled were those having lowest thresholds for surface waves; the large, phasic hair afferents from the rostral telson field of the fourth root, which have the highest probability of connecting with interneuron A (Kennedy, 1971) are normally silent under these conditions, and the fourth root afferents from the sixth segment are removed in making the preparation. Interneurons penetrated in these experiments were tested to make sure that they responded to many hairs from all over the telson field of the fourth root. Fig. 6 shows records from such

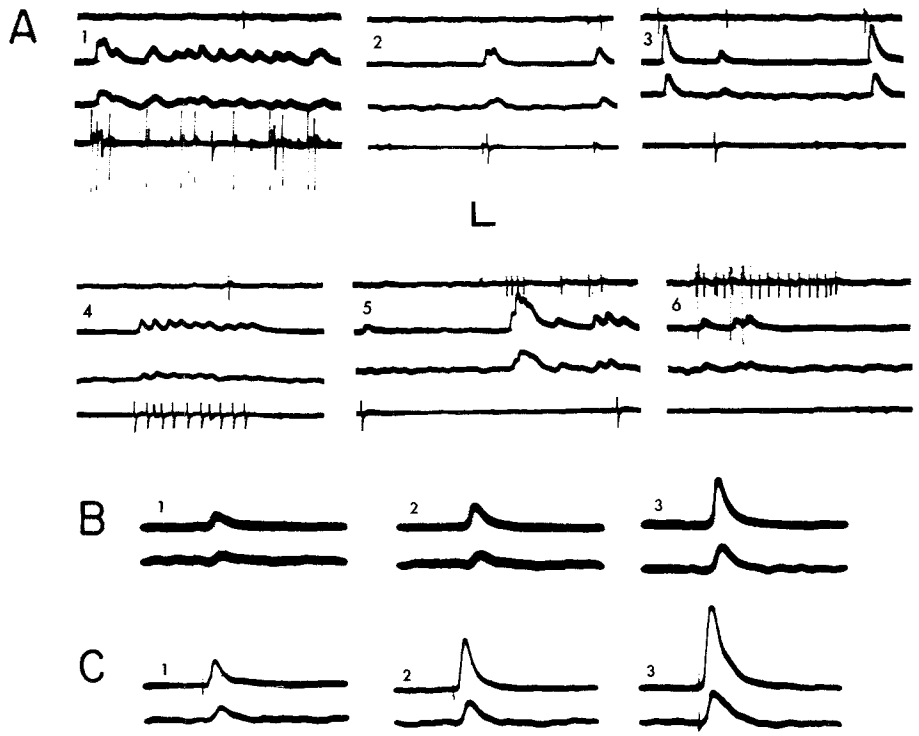

Fig. 5A—C. Simultaneous intracellular recordings from the dendrite of interneuron A and quasi-intracellular recordings from its axon in the same preparation. (A) Responses of interneuron A to mechanical stimulation of the telson. Traces are from top to bottom: ipsilateral root five afferent activity, intracellular records from interneuron A, quasi-intracellular records from interneuron A, and ipsilateral root four afferent activity. (B and C) Response of interneuron A to electrical stimulation of the ipsilateral fourth and fifth root, respectively, at three different intensities. Traces are from top to bottom; intracellular records from interneuron A, and quasi-intracellular records from interneuron A. (The ratio of intracellular to quasi-intracellular EPSP amplitude is roughly the same regardless of the root stimulated or the stimulus intensity.) Calibration bars: (A) 5 mV, 10 msec; (B, C) 3 mV, 3 msec. Voltage calibrations refer only to the intracellular traces

an experiment. Some of the identified afferents numbered 1–8 give rise to EPSP's in both interneurons, some in one and not the other, and some in neither. Table 1 shows the results of two such experiments. Experiment 1 is that of Fig. 6, and Experiment 2 compares the innervation pattern of interneuron A with that of another primary interneuron. In each case the connectivity pattern appears to be probabilistic for both postsynaptic targets: failure of a sensory axon to connect with one primary interneuron does not preclude connectivity with another. Similar results were obtained in other experiments although the number of afferents in these samples was smaller.

Interneurons with Higher-Order Connections

Many mechanoreceptive interneurons (80% of a sample of 25 interneurons from 4 preparations) encountered within the sixth ganglion showed prolonged, compound EPSP's with two amplitude peaks at different latency (bimodal) to

electrical stimulation of the fourth root (50 to over 100 msec in duration). Often these interneurons gave prolonged series of discharges, composed of 15 to greater than 20 spikes, in response to a single afferent volley. Records from such a cell—interneuron C—are compared in Fig. 7 with those from a primary interneuron, interneuron A. At low intensities of electrical stimulation of an afferent root the evoked EPSP in interneuron C was similar in waveform and duration to compound EPSP's in primary interneurons. As the stimulus intensity was increased, however, the EPSP developed a second peak (i.e., became bimodal) and became prolonged; large numbers of spikes arose from the second phase of the EPSP (Fig. 7B 2). In general it was impossible to record unitary EPSP's associated either with afferent root activity or interneuronal activity in the interganglionic connectives from these cells, however they responded to mechanical stimulation with slowly-rising depolarizations and spikes (Fig. 7A 2). Penetrations of these cells were thus probably at a distance from the actual synaptic sites; this does not explain, however, the prolonged and bimodal nature of the evoked EPSP's.

Occasionally, penetrations were closer to the synaptic sites so that unitary EPSP's associated with afferent root unit activity at short (1–3 msec) constant latency could be observed in such interneurons (Fig. 8A); these resembled EPSP's in primary interneurons in waveform, amplitude and duration (Fig. 9A) and were similarly affected by the level of polarization of the cell. In these interneurons, however, unitary EPSP's of 2–3 mV were also associated at constant latency with interneuronal discharge (Fig. 8A). Low intensity electrical stimulation of an afferent root led to a monophasic compound EPSP of relatively short duration, associated with direct afferent connections (Fig. 8B). Increases in stimulus intensity above this level caused the EPSP to become bimodal and prolonged, and unitary components of the second phase could be associated with impulses in other interneurons (Fig. 8B). Such interneurons were termed high-order interneurons; their response properties are listed in Table 2. Fig. 9 compares the latency, threshold, and time course of the response of interneuron A and interneuron C recorded simultaneously in the same preparation to electrical stimulation of an afferent root. Interneuron C responded with impulse activitiy at longer latency and at a much higher stimulus intensity than interneuron A. At high stimulus intensities interneuron C showed a much longer compound EPSP that gave rise to many more impulses.

The present results indicate that while higher-order interneurons do receive monosynaptic afferent input, interneurons also represent powerful synaptic inputs. This interneuronal input is, in fact, responsible for the prolonged EPSP's and consequently the large number of impulses observed in higher-order interneurons following brief afferent volleys.

Discussion

Sensory Axons: Roots of Entry and Central Projections

The receptive field maps of the sixth ganglion roots shown in Fig. 1 extend the observations of others (Wiersma and Hughes, 1963; Kennedy, 1971) so that now the entire mechanoreceptive surface of the crayfish abdomen is known in terms of routes of access to central ganglia. It has also been shown that telson afferents project rostrally at least to the level of the fifth ganglion. Hence,

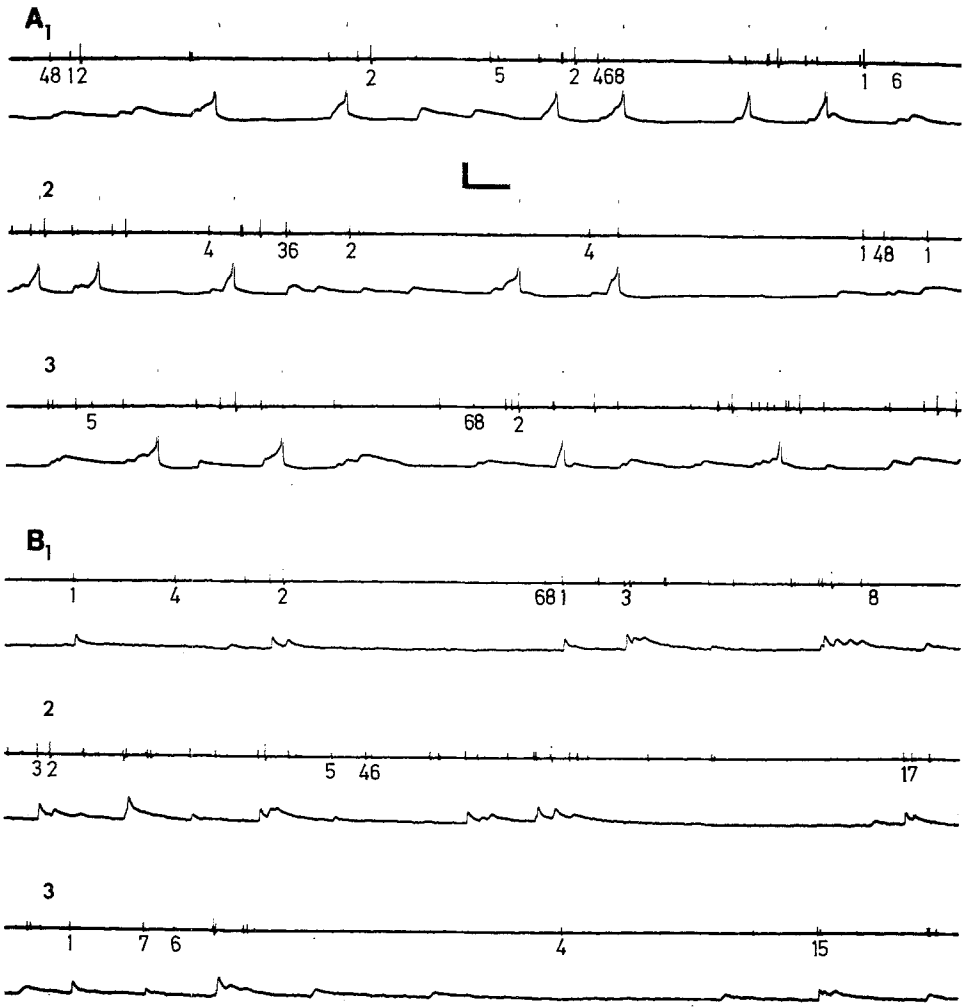

Fig. 6. Identified ipsilateral afferent input to two unidentified primary mechanoreceptive interneurons in the same preparation. In both A and B the top traces are records of fourth root afferent activity, and the bottom traces are intracellular records from the respective interneurons. Each identified afferent is numbered from 1 to 8 at least one time in both A and B except afferent 7, which is not numbered in A (see Table 1 for explanation). Afferents 6 and 8 are very small, but were readily identifiable with the aid of a magnifying glass or in afferent records at higher gain. Records are not continuous for a particular interneuron, but rather represent three exemplary stretches of record. Calibrations: (A, B) 2 mV, 10 msec. Voltage calibrations refer only to the intracellular traces

central afferent projections are probable candidates for the intersegmental summation effects previously observed in multisegmental mechanoreceptive interneurons (Kennedy and Mellon, 1964). Similarly, both an ascending and a smaller descending afferent projection have been observed for second root mechanoreceptive afferents of the third ganglion, extending the observations of Wiersma and Hughes (1961) and offering a possible explanation for the rostrally directed bias of intersegmental summation onto multisegmental mechano-

Table 1

Experiment 1

Interneuron		1	2
Afferent	1	−	+
	2	−	+
	3	+	+
	4	+	−
	5	−	+
	6	+	−
	7	?	+
	8	+	−

Experiment 2

Interneuron		1	2 (Interneuron A)
Afferent	1	+	+
	2	+	+
	3	−	−
	4	−	+
	5	−	+
	6	−	+

Data from two experiments in which serial penetrations were made of two primary mechanoreceptive interneurons in the same preparation. Exp. 1 is data from the records displayed in Fig. 6, and the Exp. 2 data is from a preparation in which interneuron A was penetrated along with another primary interneuron. Afferents are numbered in the order in which they were identified, and interneurons in the order penetrated. A + indicates that the afferent in question makes a functional excitatory connection with the indicated interneuron, Whereas a — indicates no functional excitatory connection. In Exp. 1 for interneuron 1 it was not possible to distinguish afferent 7 from afferent 1, as they have almost identical spike amplitudes and waveforms, and because neither connects with the interneuron. Therefore a ? follows afferent 7 under interneuron 1. No such trouble was encountered with interneuron 2 of the same experiment, as afferents 1 and 7 give rise to EPSP's of quite different amplitudes in this interneuron. See Fig. 6.

receptive interneurons (Kennedy and Mellon, 1964). Such central projections to adjacent ganglia are *not* made at the expense of connectivity within the ganglion of entry.

Although the cobalt techniques used to elucidate the central anatomy of fourth root mechanoreceptive afferents were somewhat less than satisfactory, the few good preparations that have been obtained probably represent accurate pictures of their central morphology. The afferent fibres appear to end within the ganglionic neuropile with little terminal branching. Although no afferent projection within the interganglionic connectives has been observed in these preparations, only the largest axons fill with cobalt; many of these, especially those associated with the phasic hairs of the rostral telson, do not project beyond the sixth ganglion. Recently, Sandeman and Okajima (1973) have filled statocyst and carapace mechanoreceptive hair afferents in the brain of the crab *Scylla* using techniques similar to those used here. These authors, too, have noted an absence of significant terminal branching in all the afferent types filled.

Both the anatomical data from cobalt-filled neurons and the electrophysiological observation that mechanoreceptive afferents project centrally only into

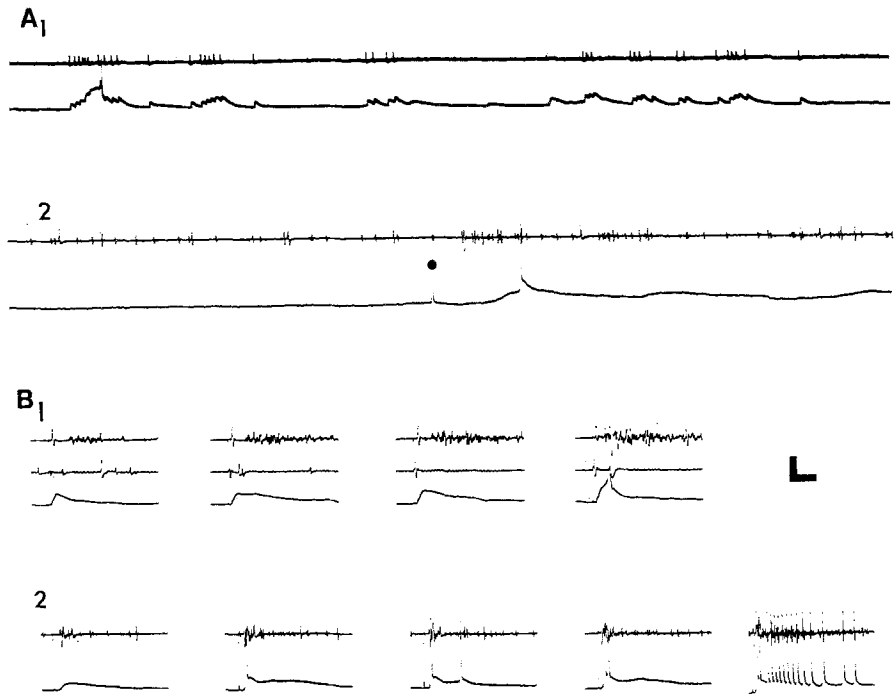

Fig. 7A—C. Comparisons of interneurons A and C. A is a primary interneuron, while C is a higher-order interneuron. (A) 1 and 2, mechanically evoked activity in the interneurons A and C, respectively. The top traces are records of ipsilateral afferent root activity, and the bottom traces are intracellular records from the interneurons. An artifact associated with the stimulus probe entering the bath is marked by a dot in 2. (B) 1 and 2, responses of the interneurons A and C, respectively, to electrical stimulation of an afferent root. The stimulus intensity increases from left to right. In 1 the traces are from top to bottom: interneuronal activity recorded in the ipsilateral interganglionic connectives, ipsilateral afferent root activity, and intracellular records from the interneuron. In 2 the top traces are interneuronal activity recorded in the ipsilateral interganglionic connectives, and the bottom traces are intracellular records from the interneuron. Each intracellularly recorded spike in interneuron C is followed by a spike recorded from its axon in the interganglionic connectives. See the text for further explanation of the figure. Calibration bars: (A) 1) 10 mV, 10 msec; 2) 5 mV, 10 msec. (B) 1) 10 mV, 10 msec; 2) 5 mV, 10 msec. Voltage calibrations refer only to the intracellular records

their ipsilateral interganglionic connective argue that the sensory axons do not cross the ganglionic midline. Similarly, statocyst and carapace hair afferents are in general restricted to their side of entry in the brain of *Scylla* (Sandeman and Okajima, 1973).

Interneuron Morphology

The cobalt impregnations presented here indicate that all or nearly all interneurons of the sixth (and fifth) ganglia that have axons in the interganglionic connectives have contralateral cell bodies. (The median cell bodies seen in Fig. 2A are mostly those of seventh root efferent fibers which have axons in the interganglionic connectives.) These results, with those of others (Kennedy, 1971; Wilkens and Larimer, 1971; Wilkens and Larimer, 1972) support the generality that crayfish interneurons have contralateral somata. It is of interest that the interneuron somata appear in two clusters in the sixth ganglion (Fig. 2A). The

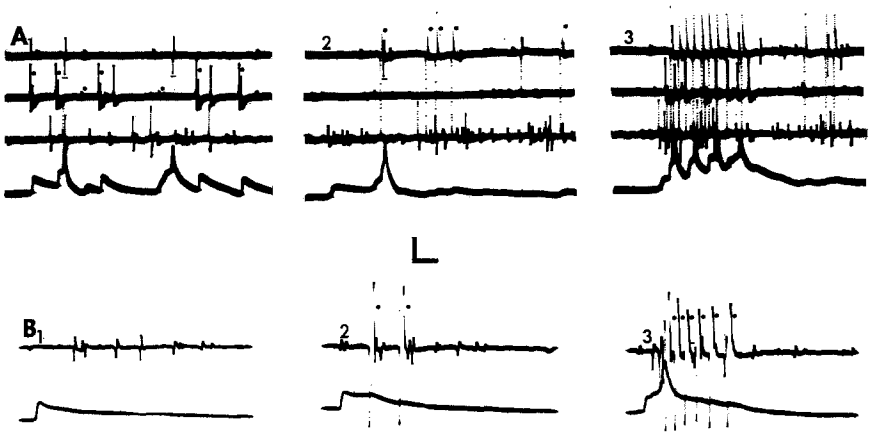

Fig. 8 A and B. Records from an unidentified higher-order mechanoreceptive interneuron. (A) Response of the cell to mechanical stimulation. Traces are from top to bottom: interneuronal activity recorded in the ipsilateral interganglionic connectives, ipsilateral afferent root activity, contralateral afferent root activity, and intracellular records from the interneuron. Dots mark each ipsilateral afferent and interneuron spike which evokes a unitary EPSP in the penetrated cell. Lines mark the spike of the interneuron recorded in the ipsilateral interganglionic connective. (B) Response of the interneuron to electrical stimulation of an ipsilateral afferent root at three increasing intensities (1 to 3, respectively). The top traces are interneuronal activity recorded in the ipsilateral interganglionic connective, and the bottom traces are intracellular records from the interneuron. Dots mark ipsilateral interneuronal spikes which add unitary components to the evoked compound EPSP's. Calibrations: (A) 10 mV, 10 msec; (B) 10 mV, 5 msec. Voltage calibrations refer only to intracellular traces

Table 2. Response properties of mechanoreceptive interneurons

EPSP's		Primary	Higher-order
Unitary			
Latency	from afferents	0.5–2 msec	1–3 msec
	from interneurons	none	not measured
Duration	from afferents	5–10 msec	5–10 msec
	from interneurons	none	5 msec or less
Amplitude	from afferents	2–10 mV	2–10 mV
	from interneurons	none	2–3 mV
Compound			
Latency		0.5–2 msec	1–3 msec
Shape		unimodal	bimodal
Duration		10–40 msec	50–> 100 msec
No. of spikes initiated		1–4	1–> 20
Latency to first spike		1–3 msec	4–5 msec
Threshold to electrical stimulation of an afferent root		low	high
% of population		20%	80%

The response properties of crayfish primary and higher order mechanoreceptive interneurons are compared. Unitary EPSP's were evoked by mechanical stimulation of hairs while compound EPSP's were evoked by electrical stimulation of afferent roots. All measurements are given as the range of observed values. Percentages of primary and higher order interneurons are derived from a sample of 25 interneurons in four preparations.

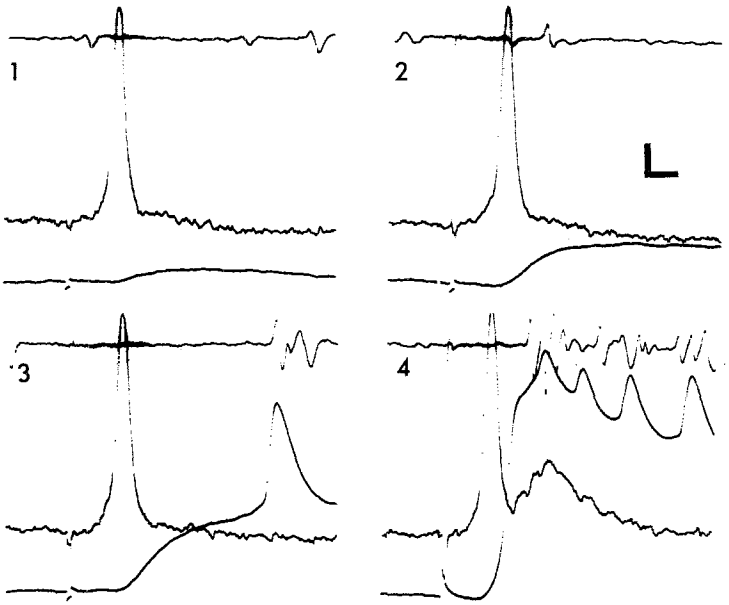

Fig. 9. Simultaneous records from interneurons A and C in the same preparation. Interneuron A was prepared for quasi-intracellular recording as described in Methods, and interneuron C was penetrated with a microelectrode. Their responses to four increasing intensities of electrical stimulation of an afferent root are shown (1 to 4, respectively). Traces are from top to bottom: interneuronal activity recorded in the ipsilateral interganglionic connective, quasi-intercellular records from interneuron A, and intracellular records from interneuron C. Calibration bars: 5 mV, 2 msec. Voltage calibrations refer only to the intracellular traces

caudal photoreceptor has its cell body in the anterior group (Wilkens and Larimer, 1972) while interneurons A (Zucker, Kennedy and Selverston, 1971) and C (Calabrese and Kennedy, 1974) have their cell bodies in the posterior group. Neither the decussation of interneurons nor their segregation into anatomical groups has, at this time, any obvious functional significance.

Evidence for Two Hierarchial Classes of Mechanoreceptive Interneurons

Table 2 summarizes the evidence that crayfish mechanoreceptive interneurons can be classified into two distinct groups based on their input properties. Using the classification scheme developed here, Wine (1975) has shown that higher order interneurons also differ from primary interneurons in that they have cell bodies which support over-shooting action potentials.

A. Primary Interneurons

A minority of the interneurons (20%) encountered within the sixth ganglion are designated as primary interneurons because under the conditions of the experiment all of their excitatory input within the sixth ganglion could be accounted for by monosynaptic chemical connections from mechanoreceptive afferents. In these cells synchronous afferent volleys give rise to short duration (usually less than 30 msec) compound EPSP's which cross firing threshold at low stimulus intensities and give rise to at most three or four impulses.

B. Higher-Order Interneurons

Most interneurons (80%) encountered within the sixth ganglion are clearly not primary in that they receive powerful input from other interneurons in addition to their direct afferent input. In these higher-order interneurons, long (50–100 msec), often prominently bimodal compound EPSP's follow synchronous afferent volleys. These compound EPSP's reach threshold only at relatively high stimulus intensities compared to primary interneurons, but because of their long duration they give rise to long trains of $\geqq 15$ spikes at high stimulus intensities. The interneuronal input to higher-order interneurons accounts for most of the spike activity observed in these cells in response to a synchronous afferent volley. Such input to higher-order interneurons can be from interneurons of the same type. (For example in Fig. 9 the large interneuron recorded—marked by dots—in the interganglionic connectives is interneuron C, a higher-order interneuron, and it provides considerable excitatory synaptic input to the penetrated interneuron.) Similarly, Zucker (1972) showed that both interneuron A, a primary interneuron, and interneuron B, a higher-order interneuron, connect synaptically with interneuron C in the third ganglion. Interneuron C also connects synaptically with interneuron B in the third ganglion (Zucker, 1972). Thus, crayfish mechanoreceptive interneurons are arranged in a hierarchy; primary interneurons receive all their input from afferents but higher order interneurons collect input from afferents, primary interneurons and other higher order interneurons.

Zucker (1972, 1973) suggested that the majority of input to mechanoreceptive interneurons was directly from afferents and relegated the role of interneuronal interaction to the synchronization of spikes in interconnected interneurons. The evidence presented here suggests, however, that such interconnection represents a major source of excitation for higher order interneurons by the addition of a positive feedback loop within the pool of interconnected interneurons that contributes heavily to burst formation. The gain of the feedback loop is less than one (individual interneuronally mediated EPSP's are small compared to the threshold of the innervated interneurons), preventing runaway bursting. The function of bursting in these cells may be related to the extraordinarily high threshold (greater than 50 mV above rest as recorded near the synaptic input sites) of the lateral giant fiber to which they are presynaptic (Zucker, 1972).

The bimodal nature of the compound EPSP induced in interneuron C and others like it (e.g. interneuron B) by electrical stimulation of an afferent root provides a simple and appealing explanation for the burst-gap-burst firing pattern often seen in these cells upon such stimulation (see Fig. 7 B 2). However, other factors may also contribute to this pattern (Kennedy and Mellon, 1964; Zucker, 1973).

The Probabilistic Nature of Mechanoreceptive Afferent Connections

The experiments on the connectivity of identified mechanoreceptive afferents with primary interneurons within the sixth ganglion show that the pattern of connection is in general probabilistic. Assessment of primary interneuronal responses to activity in a specific set of afferents by serial penetration in the same preparation demonstrated that not all sensory neurons from a particular receptor surface connect with all interneurons whose receptive fields include that surface; nor do all these interneurons receive connections from the same afferents

of that surface. It is noteworthy that missing afferents in the receptive fields of interneurons sharing a common receptor surface are not shared in common. It might have been supposed that afferents failing to connect with one interneuron did so as the result of a developmental error, or that the input element was defective in some other way. But the results clearly show that such afferents connect in the predicted way with other postsynaptic targets.

Evidence will be presented elsewhere (Wiese, Calabrese and Kennedy, in preparation) that some of the "gaps" in the receptive fields of interneurons can be accounted for on the basis of the directional nature of mechanoreceptive afferents. Most telson hairs are dually innervated, with one of the afferents sensitive to headward deflection and the other to tailward deflection. Some interneurons receive synaptic input only from the headward-sensitive afferents, and others only from tailward-sensitive ones. This segregation of afferents and interneurons cannot account for the serial penetration observations made here, however. If connectivity between afferents and interneurons is rigidly determined, and dependent on the directional properties of the afferents alone, then any pair of interneurons of the same directional class should have the same afferent "gaps" in their receptive fields; if they are of opposite directional classes, then they should share no afferents. Neither of these situations was observed. Moreover in some cases both headward-sensitive and tailward-sensitive afferents innervating a single tactile hair fail to connect with a particular directionally selective interneuron and some interneurons that show afferent "gaps" in their receptive fields (such as interneuron A) receive synaptic input from both classes of afferents (Wiese, Calabrese and Kennedy, in preparation).

In few systems can afferent connectivity be accounted for at the level of resolution attempted here. The connections of 1A muscle spindle afferents with spinal motor neurons in vertebrates (Henneman, 1974) comprise one such system, and the contrast is stark enough to warrant comparison. Every 1A afferent from a particular muscle connects with every motor neuron to that same muscle (Henneman, 1974). In the connectivity between afferents and interneurons studied here, the distribution of sensory axons is such that each interneuron receives input from a regionally varying sample of the possible afferent population; conversely, each afferent connects with a sample of the possible interneuronal population. The probabilistic nature of this pattern suggests that synaptic weighting of the various receptor surfaces that make up the receptive field of an interneuron could result from differences in the probability of connection of the interneuron with the afferents from each one. If this were so, one might expect that some interneurons would receive very few afferents from a particular receptor surface, while others would receive a larger proportion. Such observations have in fact been made (see Table 1, experiment 2).

I am particularly grateful to Dr. Donald Kennedy for advice and help with the experiments. I would also like to thank Dr. Jeffery Wine for many helpful discussions, and the technical staff of the Kennedy laboratory for their assistance.

This research was supported by a National Science Foundation Predoctoral Fellowship and Public Health Service Predoctoral Traineeship (GM 00712-16) to the author and by Public Health Service grant NB 02944 from the National Institutes of Health to Dr. Donald Kennedy.

References

Barth, F. G.: A phasic proprioceptor in the telson of the crayfish *Procambarus clarkii* (Girard). Z. vergl. Physiol. **48**, 181–189 (1964)

Calabrese, R. L., Kennedy, D.: Multiple sites of spike initiation in a single dendritic system. Brain Res. **82**, 316–321 (1974)

Harreveld, A. van: A physiological solution for freshwater crustaceans. Proc. Soc. exp. Biol. (N.Y.) **34**, 428–442 (1936)

Henneman, E.: Principles governing the distribution of sensory input to motor neurons. In: The neurosciences third study program (F. O. Schmitt, F. C. Worden, eds.) p. 281–291. Cambridge, Mass.: The MIT Press, 1974

Hughes, G. M., Wiersma, C. A. G.: Neuronal pathways and synaptic connections in the abdominal nerve of the crayfish. J. exp. Biol. **37**, 291–301 (1960)

Kennedy, D.: Crayfish interneurons. Physiologist **14**, 5–30 (1971)

Kennedy, D.: Connections among neurons of different types in Crustacean nervous systems. In: The neurosciences third study program (F. O. Schmitt, F. C. Worden, eds.) p. 379–388. Cambridge, Mass.: The MIT Press, 1974

Kennedy, D., Calabrese, R. L., Wine, J. J.: Presynaptic inhibition: Primary afferent depolarization in crayfish neurons. Science **186**, 451–454 (1974)

Kennedy, D., Mellon, DeF., Jr.: Synaptic activation and receptive fields in crayfish interneurons. Comp. Biochem. Physiol. **13**, 275–300 (1974)

Krasne, F. B.: Excitation and habituation of the crayfish escape reflex: The depolarization response in lateral giant fibers of the isolated abdomen. J. exp. Biol. **50**, 29–46 (1969)

Larimer, J. L., Kennedy, D.: Innervation patterns of fast and slow muscle in the uropods of crayfish. J. exp. Biol. **51**, 119–133 (1969)

Pabst, H., Kennedy, D.: Cutaneous mechanoreceptors influencing motor output in the crayfish abdomen. Z. vergl. Physiol. **57**, 190–208 (1967)

Pitman, T. M., Tweedle, C. D., Cohen, M. J.: Branching of central neurons: Intracellular cobalt injection for light and electron microscopy. Science **176**, 412–414 (1972)

Preston, J. B., Kennedy, D.: Integrative synaptic mechanisms in the caudal ganglion of the crayfish. J. gen. Physiol. **43**, 671–681 (1960)

Sandeman, D. C., Okajima, A.: Statocyst-induced eye movement in the crab *Scylla serrata* III. The anatomical projections of sensory and motor neurons and the responses of the motor neurons. J. exp. Biol. **59**, 17–38 (1973)

Selverston, A. I., Kennedy, D.: Structure and function of identified nerve cells in the crayfish. Endeavour **28**, 107–113 (1969)

Stretton, A. O. W., Kravitz, E. A.: Neuronal geometry: Determination with a technique of intracellular dye injection. Science **162**, 132–134 (1968)

Wiersma, C. A. G.: Giant fiber systems of crayfish. A contribution to the comparative physiology of the synapse. J. Neurophysiol. **10**, 23–38 (1947)

Wiersma, C. A. G., Hughes, G. M.: On the functional anatomy of neuronal units in the abdominal cord of the crayfish, *Procambarus clarkii* (Girard). J. comp. Neurol. **116**, 209–228 (1961)

Wilkens, L. A., Larimer, J. L.: Structural and functional morphology of two crayfish interneurons. Amer. Zool. **11**, 674–675 (1971)

Wilkens, L. A., Larimer, J. L.: The CNS photoreceptor of crayfish: Morphology and synaptic activity. J. comp. Physiol. **80**, 389–407 (1972)

Wine, J. J.: Crayfish neurons with electrogenic cell bodies: Correlations with function and dendritic properties. Brain Res. **85**, 92–98 (1975)

Wine, J. J., Krasne, F. B.: The organization of escape behavior in the crayfish. J. exp. Biol. **56**, 1–18 (1972)

Zucker, R. S.: Crayfish escape behavior and central synapses. I. Neural circuit exciting the lateral giant fibers. J. Neurophysiol. **35**, 599–620 (1972)

Zucker, R. S.: The joint peristimulus time scatter diagram is an index of the operational significance of a synapse. Brain Res. **53**, 458–464 (1973)

Zucker, R. A., Kennedy, D., Selverston, A. I.: Neuronal circuit mediating escape responses in crayfish. Science **173**, 645–650 (1971)